TRP Channels and a Dextran Sulfate Activated Rise in Intracellular Ca\textsuperscript{2+} and Na\textsuperscript{+} in a Subpopulation of Double Positive Thymocytes

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A thesis submitted for the degree of Doctor of Philosophy of The Australian National University

July 2019

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Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university. To the best of the author’s knowledge, it contains no material previously published or written by another person, except where due reference is made in the text.

Deborah Wohlfahrt

July 2019
Acknowledgements

I wish to express my deep gratitude to my supervisors Christian Stricker and Christopher Parish for their wealth of advice, kind guidance and enthusiasm throughout this project. I especially thank Christian for agreeing to supervise my rather non-neuroscience project. I believe we have both been intrigued by TRP channels and thymocytes as we delved into the field of immunology.

I also extend my sincere thanks to Harpreet Vohra and Mick Devoy for their invaluable advice and generous assistance with my flow cytometry problems, and for his valued advice and ideas on aspects of my project, I express my gratitude to Steve Daley.

To the Parish lab members - it was a fantastic opportunity to work alongside you. I enjoyed your company and music choices. I whole-heartedly thank you all, and especially David Simon Davis for his advice, assistance and patient training. Thank you to my friends in the Stricker lab, especially Le Thuy Van Tran whose insightful advice I regard most highly. I learned so much from our discussions over lunch-breaks in the sunshine and I am very grateful for her proof reading this thesis.

A special mention to my cheerleaders Lynne and Emily Raymont for their never-failing support and pep talks that kept me motivated and on track. I am also very grateful to my other family members and friends for all their encouragement and emotional support along the way.

Finally, I gratefully acknowledge the Australian National University for financial support provided by an Australian Government Research Training Program (RTP) Scholarship.
Abstract

This thesis presents the characteristics of the sustained rise of intracellular Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]\textsubscript{i}) and Na\textsuperscript{+} ([Na\textsuperscript{+}]\textsubscript{i}), observed in pre-selection CD4\textsuperscript{+}CD8\textsuperscript{+} (double-positive, DP) thymocytes when stimulated with dextran sulfate (DxS) and explores several candidate channels that may be involved in this rise. This [Ca\textsuperscript{2+}]\textsubscript{i} rise is proposed to mimic a physiological mechanism that influences early DP thymocyte selection and hence the T cell repertoire.

During thymopoiesis, the patterning of [Ca\textsuperscript{2+}]\textsubscript{i} plays a pivotal role in selection of DP thymocytes. In 1987, Tellam & Parish described such a slow and sustained Ca\textsuperscript{2+} rise in DP thymocytes which depended on a transmembrane influx of Ca\textsuperscript{2+}. The channel through which this Ca\textsuperscript{2+} entered remained elusive. Understanding the characterisation of this rise and its underlying signalling and conductance is pivotal to discovering the channel involved.

I have used flow cytometry (FACS) to concomitantly image [Ca\textsuperscript{2+}]\textsubscript{i} and [Na\textsuperscript{+}]\textsubscript{i} after DP thymocytes were exposed to 1 µg/ml DxS (500 kDa) using AM-loading of the indicators indo-1 and either ANG-2 or SBFI (5 µM for all). In some instances, Mg\textsuperscript{2+} was monitored with magflu-4. The rises were evaluated when cells were exposed to activators and blockers of receptors, cell signalling enzymes and channels. In addition, FACS-based immunocytochemistry (ICC) was employed to determine if there was channel expression on the cell surface.

In Chapter 3, I found that the earlier described Ca\textsuperscript{2+} was also accompanied by a Na\textsuperscript{+} rise. There was a delay to half-maximal amplitude of 10.8 ± 0.2 min and a rate of ~0.2 per min for both, and an amplitude of 165 ± 11 nM for Ca\textsuperscript{2+}. The one for Na\textsuperscript{+} could not be quantified but is likely in the range of several hundred µM suggesting that a non-selective cation channel is involved that predominantly
passes Na$^+$ over Ca$^{2+}$. Notably, the DxS rises are prevented by a maintained elevation of [Ca$^{2+}$]$\_i$ > 60 nM, but not by a transient rise. Additionally, the rises were absent if the recording temperature was ≤ 30°C and if [Mg$^{2+}$]$\_o$ was ≥ 25 mM.

Activated by DxS, critical elements in the signalling cascade leading to channel opening include CD8β and LFA-1 as the rises were absent in thymocytes from CD8β$^{-/-}$ and LFA-1$^{-/-}$ mice. Furthermore, activation of the Src tyrosine kinase Lck also appears essential as PP2 abolished the rises. Downstream of Lck, both PI3K and PLC activation is necessary as their inhibition by LY294002 and edelfosine, respectively, abolished the rises. The rises do not involve store release via IP$_3$ receptors and SOCE, suggesting that either PIP$_2$ hydrolysis and/or DAG generation lead to channel activation. Both disruption of F-actin polymerization and the tarantula toxin GsMTx4 abolished the rises, suggestive that mechanosensitive stimulation is involved. All these observations indicate that a non-specific cation channel of the TRP family could be involved.

In Chapter 4, commencing with a list of 14 TRP channels as candidates that share a number of the above properties, ICC and flow cytometry experiments are presented that indicate that the DAG-sensitive channels TRPC6, TRPA1 and TRPV1 are expressed and activatable in the membrane of these cells. However, none of these channels underlies the rises, as they were neither blocked by 2-APB, SKF96365, HC030031 or AMG9810, respectively. Rather, I observed that 2-APB potentiated the rises and ruthenium red abolished them, suggestive of involvement of either TRV2, TRPV3 or TRPM6. Since the rises were neither reduced by tranilast (TRPV2 blocker) nor NS8593 (TRPM6 blocker) it strongly suggests an involvement of TRPV3 in the cation rise in DP thymocytes. In support of this notion is the sensitisation by 2-APB and temperature (at 30°C) which when used independently do not cause a rise. But I cannot rule out the possibility that, upon DxS addition, the signalling cascade leading to TRPV3
activation depends on transient activation of a mechanosensitive channel such as piezo1. Such a Ca\(^{2+}\) rise, activated by a signalling cascade triggered by the CD8\(\beta\) coreceptors interacting with heparan sulfate on stromal cells, could synergistically enhance TCR signalling and fine tune selection, thus refining the repertoire of T cells.

During thymopoiesis possible inhibition of this mechanism, by fever spikes, may result in increased survival of thymocytes expressing TCRs with a high risk of self-reactivity. Importantly, progressive sialylation of developing T cells appears to negatively regulate the TRPV3 activation mechanism and so, in conditions that decrease sialylation, reactivation of TRPV3 cation influx in mature T cells is likely. Such a change might adversely increase the sensitivity to self-antigens of some T cell clones and lead to the onset of an autoimmune disorder.
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Negative selection involves large amplitude [Ca\(^{2+}\)]\(_{i}\) rises

Positive selection involves a small, cumulative [Ca\(^{2+}\)]\(_{i}\) increase

TCR–pMHC interaction duration reflects selection outcome

A SOCE mechanism triggers negative selection

SOCE is not required for positive selection

TCR–MHC independent Ca\(^{2+}\) rises

The “Tellam-Parish” Ca\(^{2+}\) rise

Overview of Ca\(^{2+}\) handling mechanisms in thymocytes

Ion channels and transporters identified in thymocytes

Voltage-gated cation channels

Voltage-independent Ca\(^{2+}\) permeable channels

Ligand-gated Ca\(^{2+}\) permeable channels

2\(^{nd}\) messenger-gated channels

Ca\(^{2+}\) activated K\(^{+}\) channels

Crucial channels in thymopoiesis

TRPM7\(^{−/−}\) causes impaired DN3 differentiation

Kv1.1 and 1.3 channels indirectly modulate Ca\(^{2+}\) entry

Cav(β2)\(^{−/−}\) causes impaired DN differentiation

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ICC detection in the PM

Activation of TRP channels in the PM

TRPA1

TRPC6

Exclusion of other candidate channels

TRPC2

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<tr>
<td>°C</td>
<td>Degree Celsius</td>
</tr>
<tr>
<td>Ab₁</td>
<td>Primary Antibody</td>
</tr>
<tr>
<td>Ab₂</td>
<td>Secondary Antibody</td>
</tr>
<tr>
<td>AF</td>
<td>Alexa fluor</td>
</tr>
<tr>
<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>ACSF</td>
<td>Artificial cerebral spinal fluid</td>
</tr>
<tr>
<td>ADPR</td>
<td>Adenosine diphosphate ribose</td>
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<tr>
<td>[Ca²⁺]ᵢ</td>
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</tr>
<tr>
<td>CaM</td>
<td>Calmodulin</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
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<tr>
<td>ConA</td>
<td>Concanavalin A</td>
</tr>
<tr>
<td>CRAC</td>
<td>Calcium release activated calcium</td>
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<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
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<td>cTECs</td>
<td>Cortical thymic epithelial cells</td>
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cTEC$^{hi}$ cortical thymic epithelial cells that highly express heparan sulfate
DAG Diacylglycerol
DN Double Negative (CD4$^-$ CD8$^-$)
DP Double Positive (CD4$^+$ CD8$^+$)
DxS Dextran sulfate 500 kDa
ECM extracellular matrix
EGTA Ethylene glycol-bis(β-aminoethyl ether)-N,N,N$'$,N$''$-tetraacetic acid
FCS Foetal calf serum
FFA Free fatty acid
Fig. Figure
FKBP FK binding protein
FITC Fluorescein isothiocyanate
HEK-293 Human embryo kidney-293 cell line
HEPES 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
HS Heparan sulfate
IC$^{50}$ half maximal inhibitory concentration of a drug
ICAM-1 Intercellular adhesion molecule 1
ICC Immunocytochemistry
ImmGen Immunological Genome Project
Indo-1 AM 2-[4-(bis(carboxymethyl)amino)-3-[2-[2-(bis(carboxymethyl) amino)-5-methylphenoxy]ethoxy]phenyl]-1H-indole-6-carboxylic acid-acetoxymethyl
IP$_3$ Inositol 1,4,5-trisphosphate
IP$_3$R Inositol trisphosphate receptor
IR-DIC Infrared-differential interference contrast
<table>
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<tr>
<td>$K_a$</td>
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<tr>
<td>kDa</td>
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<tr>
<td>LFA-1</td>
<td>Lymphocyte function-associated antigen-1</td>
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<td>MHC</td>
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<td>Mouse antibody</td>
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<td>$[\text{Na}^+]_i$</td>
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<td>$[\text{Na}^+]_o$</td>
<td>Extracellular sodium concentration</td>
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<tr>
<td>NMDG</td>
<td>N-methyl-D-glucamine</td>
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<tr>
<td>NUDT9-H</td>
<td>Nudix hydrolase motif 9-homology</td>
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<tr>
<td>OAG</td>
<td>1-Oleoyl-2-acetyl-sn-glycerol</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
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<td>PIP$_2$</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
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<td>PKC</td>
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<td>PM</td>
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<td>PLA</td>
<td>Phospholipase A</td>
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<td>PLC-$\gamma$</td>
<td>Phospholipase C gamma</td>
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<td>PLD</td>
<td>Phospholipase D</td>
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<tr>
<td>PUFA</td>
<td>Polyunsaturated fatty acid</td>
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<tr>
<td>rcf</td>
<td>Relative centrifugal force</td>
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RT  Room temperature
RuR  Ruthenium red
SAC  Stretch activated cation (channel)
SBFI AM  Sodium binding benzofuran isophthalate acetoxyethyl
SP  Single Positive
SP CD4  Single Positive CD4+ CD8-
SP CD8  Single Positive CD4- CD8+
SOCE  Store operated calcium entry
STIM  Stromal interaction molecule
TCR  T-cell antigen receptor
TM  transmembrane
TRP  Transient receptor potential
TRPA  Transient receptor potential ankyrin
TRPC  Transient receptor potential canonical
TRPM  Transient receptor potential melastatin
TRPV  Transient receptor potential vanilloid
VDCC  Voltage dependent calcium channels
$V_m$  Membrane potential
1 Introduction

1.1 Calcium as a second messenger

In all eukaryotic cells, Ca\(^{2+}\) invariably has a vital role as a second messenger molecule. By modulating the activity of Ca\(^{2+}\) binding effector molecules such as calmodulin, kinases, phosphatases and transcription factors, Ca\(^{2+}\) enables the transduction of signalling pathways that drive diverse cellular functions. These cellular processes include gene expression, cell differentiation, proliferation, cytoskeleton remodelling, motility, cell survival and cell death (reviewed by Berridge et al. 2000, Carafoli & Krebs 2016, Clapham 2007a, Lewis 2001, Samanta & Parekh 2017).

1.1.1 Ca\(^{2+}\) homeostasis

Ca\(^{2+}\) exists in cells in either free or bound form. For cell signalling, the relevant Ca\(^{2+}\) is the free form. In thymocytes, (progenitor T lymphocytes, further described in section 1.3) the cell type under investigation in this thesis, the resting free cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) is ~100–130 nM (Hesketh et al. 1983, Ishida & Chused 1988, Rogers et al. 1983, Ross et al. 1997, Tellam & Parish 1987, Tsien et al. 1982). This is approximately 10\(^4\)-fold lower than the concentration of free Ca\(^{2+}\) outside the cell ([Ca\(^{2+}\)]\(_o\) ~1.2 mM; Walser 1961), approximately 10\(^2\)-fold lower than free Ca\(^{2+}\) concentration in the endoplasmic reticulum (ER; [Ca\(^{2+}\)]\(_{ER}\) 10–250 µM; Corbett & Michalak 2000), and 5 to 10-fold lower than phosphate buffered Ca\(^{2+}\) within the mitochondrial matrix ([Ca\(^{2+}\)]\(_{mit}\) 0.5–1 µM; Nicholls 2005).

This marked concentration gradient imparts in large driving force on Ca\(^{2+}\) to move into the cytosol upon activation of Ca\(^{2+}\) permeable ion channels located in the plasma membrane (PM) and the ER membrane. Countering the rise in ([Ca\(^{2+}\)]\(_i\),
mechanisms that affect the transient binding (buffering) and removal of cytosolic Ca\textsuperscript{2+} rapidly activate. Adenosine triphosphate-dependent enzymatic pumps (ATPase), located on the PM (plasma membrane Ca\textsuperscript{2+} ATPase; PMCA) and the ER membrane (sarco-endoplasmic reticulum Ca\textsuperscript{2+} ATPase; SERCA), and ion transporters, such as sodium-calcium exchangers (NCX) and the mitochondrial Ca\textsuperscript{2+} uniporter (MCU) move Ca\textsuperscript{2+} from the cytosol back to either the extracellular compartment or into cellular organelles. Adding to these extrusion mechanisms, intracellular buffering proteins that bind Ca\textsuperscript{2+} with varying affinity further lower the free Ca\textsuperscript{2+} concentration (Milner et al. 1992). A schematic summary of the many mechanisms that may be involved in [Ca\textsuperscript{2+}]\textsubscript{i} regulation is presented in Figure 1.7 (p.77) where the role of ion channels and transporters in Ca\textsuperscript{2+} is presented in more detail.

Activation of these mechanisms differentially regulates the pattern of intracellular Ca\textsuperscript{2+} rises. Additionally, these mechanisms crucially maintain [Ca\textsuperscript{2+}]\textsubscript{i} homeostasis thereby preventing undesired [Ca\textsuperscript{2+}]\textsubscript{i} overload which is well established as cytotoxic and induces cell death (reviewed by Berridge et al. 1998, Putney 1999, Raffaello et al. 2016, Zhivotovsky & Orrenius 2011).

It is apparent therefore that the characteristics of a [Ca\textsuperscript{2+}]\textsubscript{i} rise are determined by the balance between influx, extrusion and buffering mechanisms. Influx into the cytosol via ion channels on both the PM and ER membranes is influenced by their expression (density), open probability, conductance and ion selectivity. These elements may be further modulated by their recruitment into discrete micro- or nanodomains after cytoskeletal rearrangement (Joseph et al. 2014).

Relevant to this thesis and highly dependent upon Ca\textsuperscript{2+} is the complex process of maturation of progenitor T cells (thymopoiesis) in the thymus. During thymopoiesis, diverse cellular outcomes are orchestrated by variations in the amplitude of intracellular Ca\textsuperscript{2+} and the temporal and spatial pattern of these rises.
Together, these characteristics become important determinants in the activation of signalling pathways that govern the cell fate (reviewed by Berridge et al. 2003, Christo et al. 2015, Kurd & Robey 2016).

1.2 The immune system

In higher vertebrates, the ability to defend against invading pathogens is dependent upon the development of an effective immune system, which provides both innate and adaptive immune responses. Belonging to the innate immune system, phagocytic cells, dendritic cells and natural killer cells provide early defence responses without distinguishing between microbes. Enhancing the innate system response, the adaptive immune system can trigger sophisticated and targeted responses, through activation of B and/or T lymphocytes, to defend against specific extracellular or intracellular pathogen invasion, respectively (Punt et al. 2018).

Named because they develop in the thymus, T lymphocytes can be broadly grouped according to their function. Cytotoxic T (Tc) cells recognize and mediate killing of infected and/or dysfunctional host cells. Regulatory T (Treg) cells maintain central tolerance by suppressing autoimmune activity and modulating the immune response of activated T cells. While helper T (Th) cells activate macrophages to destroy ingested pathogens, enhance Tc responses and assist in the activation of B lymphocytes, directing them to produce antigen-specific antibodies (reviewed in Bonilla & Oettgen 2010)

Relatively immature at birth, the immune system (in particular the T cell population of the adaptive immune system) undergoes rapid development and expansion in the early phase of life (Simon et al. 2015). Lying behind the sternum, the thymus provides a unique environment for the complex process of T cell development. Accommodating the production of T cells, the thymus increases in
size in early life before undergoing involution and atrophy as the rate of thymopoiesis declines with the approach of puberty (reviewed by Shanley et al. 2009). In mice a decrease in thymus size and naïve T cell output is apparent by 12 weeks after birth (Appay & Sauce 2014, den Braber et al. 2012, Sempowski et al. 2002).

1.3 Thymopoiesis

The process of T cell development begins with the migration of haematopoietic progenitor cells (HPCs) from the bone marrow to the thymus. As illustrated in Figure 1.1A, the HPCs enter the thymic environment at the corticomedullary junction and begin their complex process of differentiation, proliferation and selection with the mature progeny (naïve T cells) egressing to the periphery two to three weeks later (Egerton et al. 1990). As they randomly migrate outward from their entry point, through distinct stromal regions to reach the subcapsular region (SCR), the HPCs proliferate and differentiate through four sequential “double negative” (DN) stages. This naming convention indicates that the CD4 and CD8 lineage markers are not yet expressed (negative) on these cells. Rather, the DN1–DN4 stages are defined by the differential expression of other surface receptors such as CD25, CD44 and pre-T cell antigen receptor (TCR) molecules (shown in Figure 1.1B). During their migration through the 3-dimensional network of varying thymic microenvironments, which takes about two weeks, the developing thymocytes dynamically interact with diverse stromal cells. Their exposure to varying endogenous ligands, including neuropeptides, lipids, adhesion molecules and hormones (Juzan et al. 1992, Rousseau et al. 2015) provides crucial developmental signals that induce activation of transcription factors and gene expression.
Figure 1.1 Thymopoiesis in a thymic lobe

A. Haematopoietic progenitor cells (HPCs) enter the thymus at the corticomedullary junction (CMJ). Migrating through the cortex to the subcapsular zone, they differentiate through four distinct double negative stages (DN1 - 4). DN4 thymocytes that differentiate to become double positive (DP) thymocytes express both the CD4 and CD8 coreceptor molecules and a T cell receptor complex. Interaction with thymocyte stromal cells including cortical thymic epithelial cells (cTECs), dendritic cells, macrophages and medullary TECs (mTECs), provides stimuli which direct further selection processes. Thymocytes that pass positive selection continue lineage differentiation to become either SP CD8 or SP CD4 cells. Upon further testing, SP thymocytes that are not negatively selected egress the thymus as naïve CD4 or CD8 T cells. B. Thymocyte developmental stages. The expression of the CD4 and CD8 coreceptor molecules distinguishes
double positive (DP) thymocytes. Preceding DP development are four double negative (DN) stages that can broadly be distinguished by expression of CD44 and CD25. Critical check points (red text) include T lineage commitment, TCRγδ rearrangement leading to differentiation of γδ T cells, β selection and MHC or CD1d restriction. Distinct from CD4SP and CD8SP thymocytes invariant natural killer T (iNKT) cells engage with MHC-1 like molecule CD1d and become DN.

Not unexpectedly, different Ca²⁺ rises are critical to the transduction of many of these signals. For instance, specific developmental arrests and altered autophagy occur in DN thymocytes deficient in the Ca²⁺ permeable transient receptor potential (TRP) channels TRPM7 (Jin et al. 2008a) and TRPV1 (Farfariello et al. 2012), respectively. In addition, an altered Ca²⁺ influx observed in phospholipase C-γ 1 (PLC-γ1) deficient thymocytes disturbs differentiation from the DN3 to DN4 stage (Fu et al. 2017).

1.3.1 The αβ TCR/CD3 signalling complex

At the DN3 stage, precursor thymocytes must pass a critical check point, known as β selection (Figure 1.1B). This check point ensures the newly formed pre-TCR (comprising a β chain and a pre-Tα chain) is functional (Carpenter & Bosselut 2010). Those that pass, continue to differentiate and proliferate to form a large pool of DN4 thymocytes which express a diverse range of nascent αβ TCRs.

Depicted in Figure 1.2, the α and β chains that form the αβ TCR comprise a proximal constant (c) domain and a distal variable (v) domain. Somatic rearrangement of three gene segments (V, D and J) during development of the α and β chains results in the expression of variable domains that have diverse amino acid sequences (Davis & Bjorkman 1988). The resulting structure determines the affinity of the TCR for peptides/antigens presented on major histocompatibility complex (MHC) molecules (Bonilla & Oettgen 2010). Associated with the αβ TCR are CD3 ε, δ, γ and ζ protein chains that form εδ, εγ and ζζ dimers. Altogether, these form the TCR/CD3 signalling complex as shown in Figure 1.2. DN thymocytes that survive through to terminal differentiation,
migrate from the subscapular zone back through the cortical region, differentiating to become preselection-double positive (DP) thymocytes (expressing both CD8 and CD4 coreceptors).

As indicated by their TCR\textsuperscript{lo}CD4\textsuperscript{+}CD8\textsuperscript{+}CD69\textsuperscript{low}CD5\textsuperscript{low} phenotype, preselection DP thymocytes have significantly lower surface expression of αβTCR (Guidos et al. 1990) and markers of thymocyte activation (CD69 and CD5; Bhandoola et al. 1999) compared to more mature DP and SP thymocytes. However, a heightened sensitivity to TCR stimulation observed in preselection thymocytes enables their activation by low affinity peptide binding. This enhanced response is in part due to participation of the coreceptors and the proposed regulation by the microRNA miR-181a (Davey et al. 1998, Li et al. 2007).

Depicted in Figure 1.2, the role of the CD8 and CD4 coreceptors is to bind to the MHC class I (MHC-I) and MHC class II (MHC-II) molecules, respectively, and facilitate delivery of the tyrosine kinase Lck to the TCR/CD3 complex (Artyomov et al. 2010, Jiang et al. 2011, Li & Mariuzza 2013, Stepanek et al. 2014, Turner et al. 1990). In DP thymocytes, Lck is highly expressed and importantly associated with the cytosolic C-terminal of CD4 and to a lesser extent the CD8 coreceptors (Wiest et al. 1993). In thymocytes, sequestration of Lck to the coreceptor molecules has been proposed as a mechanism for ensuring TCR signal activation remains specific to MHC interactions (Van Laethem et al. 2007). However, Stepanek et al. (2014) argue that very few coreceptors actually couple Lck and that recruitment of coreceptors coupled to Lck to the TCR/CD3 complex provides a rate-limiting step in TCR/CD3 signal initiation.
Figure 1.2 Illustration of the αβTCR/CD3 complex–pMHC interactions

Ready for “testing” the newly arranged αβTCR/CD3 complex includes the TCR α and β chains and the CD3 signalling molecules which comprise the γ, δ, ε and ζ chains. The TCR α and β chains both contain variable antigen recognition (v) domains and invariant/constant (c) signalling domains. (A). The interaction of the peptide (p)MHC-I complex with the αβTCR is stabilised by CD8 coreceptor binding to MHC-I. (B). CD4 coreceptor binding to MHC-II enhances αβTCR–pMHC-II interactions during T lymphocyte interaction with APCs. Coupled to the cytosolic tail of CD4 and CD8 is the tyrosine kinase Lck.

1.3.1.1 MHC–αβTRC engagement

Selection of DP thymocytes is dependent upon αβTCR recognition of MHC polymorphic residues in the binding domain (MHC restriction) and on the avidity of peptide engagement (Matzinger et al. 1984, Winchester 2008). Expressed on all nucleated cells, MHC-I molecules bind peptide fragments (p) derived from within the cell (pMHC-I) which they present to αβTCRs. This interaction is stabilized by CD8–MHC-I ligation at a site distinct from the αβTCR recognised pMHC-I binding domain (Borger et al. 2014). Different from MHC-I, MHC-II molecules are found on antigen presenting cells (APCs) such as macrophages, dendritic cells, B cells and thymic epithelial cells (TECs). Aided by CD4 coreceptor binding, the pMHC-II complex presents peptide fragments
derived from the extracellular milieu to αβTCRs. Associated with stimulation of the αβTCR by pMHC binding (reviewed by Moogk et al. 2018), the coreceptor-facilitated Lck phosphorylation of the CD3 chains initiates signal transduction. Subsequent recruitment of ζ-chain-associated protein kinase of 70 kDa (ZAP-70) to CD3 increases phosphorylation of the exposed CD3 immunoreceptor tyrosine-based activation motif domains (ITAMs) and amplifies the αβTCR/CD3 signalling cascade (reviewed by Bonilla & Oettgen 2010, Lee et al. 2015, Palacios & Weiss 2004, Rossy et al. 2012).

Engagement of the MHC by the co-receptor molecules augments the stability of αβTCR–pMHC interaction and influences the bond lifetime thereby fine tuning TCR/CD3 reactivity to presented peptides (Hong et al. 2018). Furthermore, the CD8–MHC-I engagement has been proposed to facilitate αβTCR–pMHC dependent conformational change in CD3ε (Gil et al. 2008) and enhance Lck delivery to the TCR/CD3 complex (Davey et al. 1998, Delon et al. 1998, Hong et al. 2018, Lucas et al. 1999, Palmer & Naeher 2009, Stepanek et al. 2014, Thome et al. 1996). In preselection DP thymocytes, the low sialylation state of the CD8 coreceptor markedly enhances CD8–MHC-I binding and its role in MHC-I–TCR/CD3 activation (Gil et al. 2008, Moody et al. 2001). However, in the absence of αβTCR–pMHC-I interaction, Grebe et al. (2004) suggested that CD8–MHC-I ligation may induce apoptosis in preselection DP thymocytes.

At the DP stage, rigorous testing of the newly formed αβTCR commences to ensure selection of thymocytes that fulfil two criteria. Firstly, they express TCRs that are restricted to binding MHC molecules. Secondly, the binding affinity of the αβTCR to pMHC is both sufficient and self-tolerant. The vast combinatorial diversity in the v domains of the αβTCR chains (approximately 10^{15} permutations; Davis & Bjorkman 1988) means there will be receptors that are not necessarily restricted to binding to MHC molecules and/or receptors that bind
too strongly to MHC molecules. Overall, the “pass rate” of thymocytes is exceedingly low as most thymocytes undergo apoptosis within the thymus (Egerton et al. 1990). Approximately 65% of preselection DP thymocytes are predicted to “die by neglect” due to inadequate αβTCR–pMHC binding. In the thymic cortex, this failure to be positively selected is the primary cause of DP thymocyte death (Surh & Sprent 1994, Szondy et al. 2012). Of those remaining, a further 91% are likely to undergo apoptosis as a result of negative selection triggered by high affinity αβTCR–pMHC binding (Sawicka et al. 2014). While apoptosis more commonly occurs in the medullary region (reviewed by Starr et al. 2003b), strong αβTCR–pMHC binding will induce negative selection of DP thymocytes within the thymic cortex (Murphy et al. 1990, Surh & Sprent 1994).

1.3.1.2 Thymic epithelial cell interaction triggers selection signals

As DP thymocytes randomly migrate through the thymic cortex, they intermittently stop to interact with cortical TECs (cTECs) to receive crucial TCR activation signals which drive positive as well as early negative selection (discussed by Palmer & Naeher 2009). These necessary interactions vary in duration and frequency, from approximately two to four min every 30 min for interactions leading to positive selection (Ross et al. 2014) to prolonged interactions of >20 min linked to negative selection (Melichar et al. 2013; further described in section 1.4; Figure 1.5). Notably, the intensity of the TCR response influences the amplitude and the patterning of the associated [Ca\(^{2+}\)]\(_i\) rises (Davey et al. 1998). Fluctuations in cytosolic Ca\(^{2+}\) ranging from 0.3 to 1 µM have been described (Bhakta & Lewis 2005, Bhakta et al. 2005, Melichar et al. 2013, Melichar et al. 2015, Ross et al. 2014). While it is generally agreed that the variation in [Ca\(^{2+}\)]\(_i\) is critical in the complex processes of positive and negative selection (reviewed in Feske 2007, Feske et al. 2015, Vig & Kinet 2009), understanding of the
mechanisms underpinning these \([\text{Ca}^{2+}]\) rises (reviewed in section 1.4) remains incomplete.

Mentioned previously, surface expression of the \(\alpha\beta\) TCR in DP thymocytes is about ten-fold lower than that of more mature SP thymocytes (reviewed by Schrum et al. 2003). However, in DP thymocytes the \(\alpha\beta\) TCRs are more reactive to low affinity peptides (Davey et al. 1998). This sensitisation of the \(\alpha\beta\) TCR–pMHC interactions, which promotes positive selection, is in part facilitated by the CD4 and CD8 coreceptors (Gil et al. 2008, Moody et al. 2003, Nakayama et al. 1990, Simon Davis 2015, Starr et al. 2003a), and by the activation of adhesion molecules such as the lymphocyte function-associated antigen-1 (LFA-1) and CD2. These interact dynamically with their respective endogenous ligands expressed on cTECs (Nonoyama et al. 1989, Paessens et al. 2008). Recognised as adhesion molecules, contemporaneous activation of LFA-1 and CD2 during thymocyte–cTEC interaction may synergistically add to the \([\text{Ca}^{2+}]\) rises and thereby critically affect the outcome of selection (Danielian et al. 1992, Ohno et al. 1991, Revilla et al. 1997, Teh et al. 1997).

1.3.1.3 Enhancement of TCR/CD3 signalling by LFA-1

Highly expressed on thymocytes (Fine & Kruisbeek 1991), LFA-1 comprises the \(\alpha\) and \(\beta\) chains, CD11a and CD18 respectively (Ma et al. 2002), which adopt an “inactive” conformational state at rest. In thymocytes, activation of LFA-1 mediates binding to TECs (Singer et al. 1990) and significantly biases the commitment to the CD8\(^+\) T lymphocyte lineage (Revilla et al. 1997). In vivo saturation of the CD11a chain by anti-LFA-1\(\alpha\) mAb injection was found to selectively decrease the population of CD8 SP thymocytes in treated mice, suggesting that LFA-1 plays a crucial role in the generation of these thymocytes. Perhaps the mechanism by which LFA-1 activation enhances selection is like that which has been described in mature freshly isolated CD8 T cells. In these cells,
prior activation of LFA-1 by adherent antibodies, raised against its CD11a or CD18 chains “primed” the strength of the transduced TCR signal (activated by soluble pMHC monomers) and augmented the subsequent [Ca\textsuperscript{2+}] rise (Randriamampita et al. 2003).

Under physiological conditions, that is, at 37°C but not 4°C (Moingeon et al. 1991), the mechanism activating LFA-1 follows early signalling events downstream of TCR/CD3 activation (Mueller et al. 2004). Under tight regulation, the extracellular domain of LFA-1 naturally adopts a folded conformation with low affinity for its major endogenous ligand, intercellular adhesion molecule 1 (ICAM-1). To adopt an activatable extended conformation, LFA-1 requires an inside-out signal (Cairo et al. 2006). In thymocytes, the precise molecular mechanisms that regulate LFA-1 activation remain to be characterised. However, studies that investigated the mechanism in Jurkat or mature T lymphocytes suggest that the transduction of the inside-out signal is mediated by at least 4 mechanisms. These are 1) CD4/CD8–Lck kinase activation (Fagerholm et al. 2002, Vielkind et al. 2005), 2) CD3ε conformational change that allows recruitment of adaptor proteins including the non-catalytic region of kinase domain (Nck; Lettau et al. 2014), the SH2 domain-containing leukocyte protein of 76 kDa (SLP-76; Horn et al. 2009), the adhesion and granulation-promoting adaptor protein (ADAP; Wang et al. 2009, Wu et al. 2006) and the Wiskott-Aldrich syndrome protein (WASP); 3) F-actin remodelling regulated by calpain (a Ca\textsuperscript{2+} dependent protease, Stewart et al. 1998) activity and talin an integrin–actin binding protein (Cairo et al. 2006, reviewed by Hogg et al. 2011, Kim et al. 2003, Lub et al. 1997) and 4) increased cytosolic Ca\textsuperscript{2+} (Guo et al. 2018, Liu et al. 2018, van Kooyk et al. 1993). Such a signalling mechanism is shown in Figure 1.4 (p. 51) where these signalling molecules are proposed to be activated during thymocyte–cTEC interaction without αβTCR–MHC engagement.
Once in an extended conformation, LFA-1 activation by ICAM-1 or by anti-LFA-1 Ab triggers a complex outside-in signalling cascade. Also not well characterised in thymocytes, the transduction of the outside-in signal in other T lymphocytes reportedly entails 1) Src kinase phosphorylation of adaptor proteins including C10 regulator of kinase (Crk; Roy et al. 2018), ADAP (Suzuki et al. 2007, Wang et al. 2009), SLP-76 (Baker et al. 2009); 2) localised formation of “actin-clouds” (Suzuki et al. 2007); 3) recruitment and activation of focal adhesion kinase (FAK) and proline-rich tyrosine kinase 2 (PYK-2; Rodriguez-Fernandez et al. 1999); and 4) enhanced activation of effector molecules including Vav, a guanine nucleotide exchange factor that crucially regulates actin polymerization (Kong et al. 1998), phosphoinositol 3 kinase (PI3K) and PLC-γ1 (Kim et al. 2009a, Sánchez-Martín et al. 2004).

Activation of LFA-1 signal transduction is proposed to facilitate TCR activation. In conditions of suboptimal TCR stimulation, Kim et al. (2009a) showed that LFA-1 interaction with ICAM-1 resulted in a $[\text{Ca}^{2+}]_i$ rise (Figure 1.3A, black trace) that involved PLC-γ1 activation, but was independent of store operated Ca$^{2+}$ entry (SOCE). This $[\text{Ca}^{2+}]_i$ rise was abolished in WT cells preincubated with anti-LFA-1 mAb and in LFA-1$^+$ T cells (Figure 1.3A, orange and green, respectively). Additionally, Kim et al. (2009a) showed that the LFA-1 enhanced Ca$^{2+}$ influx was abolished by inhibiting the Src kinase and significantly reduced following pre-treatment with the PI3K and PLC-γ1 inhibitors, respectively. Without TCR stimulation, they found the addition of anti-LFA-1 mAb was not sufficient to evoke a $\text{Ca}^{2+}$ rise (Figure 1.3A, grey trace), most likely because LFA-1 remained in a folded inactive state. They conclude that essentially in conditions of suboptimal TCR stimulation, synergistic signal transduction via activated LFA-1 augments activation of PLC-γ1 and PI3K signalling cascades and increases the $[\text{Ca}^{2+}]_i$ rise. This synergistic $[\text{Ca}^{2+}]_i$ rise potentially lowers the threshold for TCR signalling activation (Wang et al. 2008, Wulfig et al. 1998). In DP thymocytes
such a mechanism could promote positive selection downstream of poor avidity pMHC–TCR interaction.

1.3.1.4 TCR/CD3 signalling may be enhanced by CD2

Further to LFA-1 enhancement of T-lymphocyte signalling, an additive \([\text{Ca}^{2+}]_i\) rise associated with CD2 activation perhaps negatively regulates DP thymocyte sensitivity to TCR positive selection signals (Bierer et al. 1988, Teh et al. 1997). Distinct from LFA-1 activation, in T lymphocytes, CD2 adhesion and signal transduction activity have been shown to be independent of TCR signalling, \([\text{Ca}^{2+}]_i\) and temperature (Meuer et al. 1984, Moingeon et al. 1991). Independent of TCR stimulation in T lymphocytes, cross-linking anti-CD2 mouse antibody (mAb) has been shown to evoke a sustained \([\text{Ca}^{2+}]_i\) rise (Figure 1.3B) that comprised both intracellular and extracellular components, suggestive of activation of a SOCE mechanism (Ledbetter et al. 1988, van Kooyk et al. 1993).

In Jurkat cells, ligation of CD2 to the endogenous glycoprotein CD48 (mice; CD58/LFA-3 humans) commonly expressed on cTECs and APCs, has been shown to promote signal transduction via activation of protein tyrosine kinases including Fyn, Pyk2 (Sunder-Plassmann & Reinherz 1998), and spleen tyrosine kinase (Syk; Umehara et al. 1998). These then lead to an \([\text{Ca}^{2+}]_i\) rise (Bachmann et al. 1999, Vollger et al. 1987). Although preselection DP thymocytes express lower levels of CD2 than mature thymocytes (Duplay et al. 1989), its colocation with LFA-1 (Altin et al. 1994) perhaps enables the synergistic activation of Lck, Fyn and Syk (Danielian et al. 1992, Lin et al. 1998) and enhanced recruitment and activation of PI3K and PLC-γ1 (Dietsch et al. 1994, Espagnolle et al. 2007, Kivens et al. 1998, Shimizu et al. 1995). There is evidence that thymopoiesis is subtly altered in CD2− mice. Sasada and Reinherz (2001) found in these mice that the T cell repertoire of the peripheral pool was decreased compared to that of the WT mice and they proposed that this change reflected altered thymocyte selection.
However, this disruption of thymopoiesis appeared to precede DP differentiation, and whether CD2-dependent parallel signalling has a role in directing TCR/CD3 signalling in preselection DP thymocytes remains unknown.

1.3.1.5 αβ TCR reactivity modulated by glycosylation of CD8

In the thymus the variable expression and secretion of glycosaminoglycans (GAGs) by different stromal cell types is likely to support the formation of distinct microenvironments that facilitate thymocyte maturation (Simon Davis 2015, Werneck et al. 2000, Werneck et al. 1999). GAGs are linear polysaccharide chains which often covalently attach to a core protein, forming a large family of proteoglycans. Found intracellularly, on cell surfaces and in the extracellular matrix, these structurally diverse and sometimes highly sulfated macromolecules are essential to normal cell function (reviewed by Bishop et al. 2007, Gandhi & Mancera 2008). Perhaps the best studied GAG is the ubiquitously expressed heparan sulfate (HS).

1.3.1.5.1 Heparan sulfate

Covalently bound to a core glycoprotein, HS is proposed to have a role in multiple responses in the immune system, including the modulation of the TEC–thymocyte adhesion (Werneck et al. 1999), the initial expression of the TCR, CD4 and CD8 coreceptors in early thymocyte development (Wrenshall et al. 1993), immune cell migration and their activation (reviewed by Simon Davis & Parish 2013). The polyanionic nature of HS enables multiple interactions with many classes of proteins. Such interactions are commonly electrostatic in nature (Ori et al. 2011). In their comprehensive study of the rat HS interactome, Ori et al. (2011) conclude that heparan sulfate binding proteins (HSBPs) are strongly associated with transduction of extracellular cues to intracellular signalling pathways that lead to a diverse range of biological responses, including cell adhesion and
possible modulation of the kinetics of some ion channels. Specifically, in lymphocytes, 234 candidate HSBPs have been proposed based on the detection of a “consensus” HS-binding motif. Of these candidates, 54 are found in the PM (Simon Davis & Parish 2013). Notably, in DP thymocytes, increased mRNA expression has been described for some candidate HSBPs. These include the coreceptor chains CD8β, CD4, the protein tyrosine phosphatase receptor CD45, and an adhesion G-protein coupled receptor E5 (CD97). A list of these, their proposed function and the mRNA expression level in DP thymocyte subpopulations is provided in Appendices 1 and 2, respectively (pp. 349 & 351).

1.3.1.5.2 **Dextran sulfate (DxS)**

Just as extracellular GAGs interact with and influence activation of cell surface receptors and membrane lipid dynamics (Huster et al. 1999, reviewed by Sahoo & Schwille 2013), for many years it has been recognised that high molecular weight (mol. wt.) sulfated polysaccharides, such as DxS, may also trigger cellular responses in lymphocytes (Palacios et al. 1982, Sasaki & Suchi 1967, Sugawara & Ishizaka 1982). The addition of 0.1 mg/ml DxS (50 kDa) has been shown to promote mitogenic activity in mouse peripheral T cells, but notably the presence of irradiated autologous adherent cells was necessary (Palacios et al. 1982). Relevant to this thesis, it is assumed that DxS likely cross-links with PM proteins which results in the subsequent activation of HSBPs, most crucially CD8β, and perhaps with other polyanionic binding sites, such as those identified on CD4 (Lederman et al. 1989, Parish et al. 1990), CD2 (Warren & Parish 1990) and LFA-1 (Vermot-Desroches et al. 1991).

1.3.1.6 **HS causes rosetting on cTECs in preselection DP thymocytes**

HS proteoglycans (HSPGs) were found to be variably abundant on cell surface extracts purified from cTEC like cells (Werneck et al. 1999). More specifically, in
thymic slices prepared from C57BL/6 mice, Simon Davis (2015) identified multiple “hot spots” stained with an anti-HS mAb suggesting a high local concentration of HS. Restricted to the thymic cortical region, these focal cTECs (hereafter described as cTEC\textsuperscript{hi}) had about a 20-fold higher anti-HS mAb binding compared to other cTECs (Simon Davis 2015). It is hypothesised that HS on these cTECs binds to CD8 coreceptors on preselection DP thymocytes at a location distinct from the MHC-I binding site. This HS–CD8 binding in addition to MHC promotes the formation of stable rosette-like structures, where multiple thymocytes form clusters with a single cTEC (Klewski et al. 1982, Oliveira-dos-Santos et al. 1998). Further supporting a role for CD8, the ability to form stable thymocyte–cTEC\textsuperscript{hi} rosettes was markedly impaired in CD8α\textsuperscript{-/-} and CD8β\textsuperscript{-/-} thymocytes (Simon Davis 2015).

In a series of elegant experiments, DP thymocyte–cTEC\textsuperscript{hi} congregates (described as rosetting) have been experimentally formed and the associated Ca\textsuperscript{2+} rises quantified using flow cytometry (Simon Davis 2015). Consistent with earlier findings (Hare et al. 2003), Simon Davis (2015) found rosette formation was significantly enhanced by αβTCR–pMHC engagement. Specifically, the stability and number of rosettes was markedly higher in samples where thymocytes were mixed with WT cTECs, compared to those where thymocytes were mixed with MHC-I/II deficient cTECs. Furthermore, a significant and sustained [Ca\textsuperscript{2+}]\textsubscript{i} rise was observed in DP thymocytes rosetting on WT cTECs compared to non-rosetting thymocytes (Figure 1.3C; black and grey, respectively). Significant to this thesis, in the absence of TCR–MHC-I/II binding, while reduced, rosette formation was not completely abolished (Figure 1.3C; red) compared to non-rosetting thymocytes (Figure 1.3C; pink). The formation of rosettes under these conditions was associated with a small amplitude [Ca\textsuperscript{2+}]\textsubscript{i} rise which reached a plateau after about 10 min, suggesting the existence of an alternate [Ca\textsuperscript{2+}]\textsubscript{i} source. Independent of TCR–MHC-I/II interaction, this [Ca\textsuperscript{2+}]\textsubscript{i} rise was possibly evoked
by the activation of surface receptors, notably CD8 and perhaps the integrins LFA-1 and CD2 upon binding to the respective ligands expressed on cTECs (Kanner et al. 1993, Simon Davis 2015). While the role for CD2 in enhancing preselection DP–cTEC<sup>hi</sup> binding has not been determined, there is evidence that CD2 plays a significant role in thymocyte–erythrocyte autorosetting.

While a mechanism of enhanced signal transduction evoked by HS–CD8β chain binding remains to be identified, it is notable that independent of TCR–pMHC engagement, the CD8β chain has been found to physically interact with the CD3δ and to a lesser extent CD3ε and γ chains (Gil et al. 2008, Suzuki et al. 1992). This interaction is proposed as a mechanism for inducing a conformational change of CD3 and therefore an important early step in signal transduction and positive selection (reviewed by Bettini et al. 2014, Ma et al. 2017, Xu et al. 2008). Whether this conformational change requires an increase in [Ca<sup>2+</sup>] in the TCR/CD3 microdomain and precedes CD3 phosphorylation remains a topic of debate (Gagnon et al. 2012, Gil et al. 2005, Shi et al. 2013).
Figure 1.3 [Ca\textsuperscript{2+}]\textsubscript{i} rises induced by costimulatory receptor activation

Time courses of [Ca\textsuperscript{2+}]\textsubscript{i} rises evoked in thymocytes in conditions of suboptimal TCR activation.

(A) Ca\textsuperscript{2+} responses in CD8\textsuperscript{+} T lymphocytes following the addition of plasma membrane vesicles expressing the endogenous ligand ICAM-1 and peptide loaded MHC molecules (arrow). The [Ca\textsuperscript{2+}]\textsubscript{i} rise is observed in WT T lymphocytes (black), but not when the cells were LFA-1\textsuperscript{-/} T lymphocytes, or treated with anti LFA-1Ab or where the peptide did not bind to the TCR (green, orange and grey respectively; adapted from Kim et al. 2009a).

(B) [Ca\textsuperscript{2+}]\textsubscript{i} rise observed in T cells incubated for 5 min with mAb against CD2 and cross-linked by goat anti-mouse Ig Ab at 1.5 min (adapted from Ledbetter et al. 1988).

(C) Monitoring of [Ca\textsuperscript{2+}]\textsubscript{i} in preselection DP thymocytes mixed with cTEC\textsuperscript{hi}. WT thymocytes rosetting on WT cTEC\textsuperscript{hi} show a sustained [Ca\textsuperscript{2+}]\textsubscript{i} rise (black) compared to non-rosetting ones (grey). [Ca\textsuperscript{2+}]\textsubscript{i} rise in WT thymocytes rosetting on MHC-I/II\textsuperscript{-} cTECs (red) and in non-rosetting ones (adapted from Simon Davis 2015).

(D) The addition of 1 µg/ml DxS (arrow) to a thymocyte suspension evokes a delayed [Ca\textsuperscript{2+}]\textsubscript{i} rise that is specific to DP thymocytes (adapted from Tellam & Parish 1987).

Investigating the characteristics of thymocyte autorosetting (Parish et al. 1984) reported that rosetting receptors also bound with high affinity to sulfated polysaccharides such as heparin. Preincubation with fucoidan and dextran sulfate competitively prevents this phenomenon. Adding to this observation, Simon
Davis (2015) found rosetting between cTEC<sub>hi</sub>–preselection DP thymocytes was also significantly inhibited by prior addition of dextran sulfate or HS mimetics like heparin to the cell suspension. It is likely these HS mimetics and DxS competitively bind to rosetting receptors, preventing HS interaction with HSBPs, in particular CD8. Furthermore, dependent upon low CD8 sialylation (Simon Davis 2015), the addition of DxS<sub>500 kDa</sub> (DxS) to a thymocyte suspension evokes a [Ca<sup>2+</sup>] rise (as shown in Figure 1.3D, adapted from Tellam & Parish 1987). First observed in 1987, this DxS [Ca<sup>2+</sup>] rise was found to be specific to DP thymocytes (Tellam & Parish 1987, Weston et al. 1991). In this thesis, it is postulated that the cross-linking of multiple surface receptors, including CD8, by DxS, mimics an endogenous mechanism activated in preselection DP thymocytes during their interaction with cTEC<sub>hi</sub>. Because this observation is central to the research presented in this thesis, the characteristics of this DxS [Ca<sup>2+</sup>] rise are reviewed in greater detail in section 1.5.

1.3.1.6.1 Proposed DP thymocyte–cTEC<sub>hi</sub> signalling mechanisms

For some early selecting DP thymocytes, cTEC<sub>hi</sub> rosetting may provide a mechanism by which selection signalling pathways are adjusted consequent to [Ca<sup>2+</sup>] rises and that is dependent upon HS evoked CD8β chain activation. During preselection DP thymocyte–cTEC<sub>hi</sub> interaction, numerous surface receptors are most likely activated as they engage their endogenous ligands. Considering just CD8–HS binding, physical interaction between CD8β and CD3 and binding of the adhesion molecules LFA-1 and CD2 to ICAM-1 and CD48 respectively, Figure 1.4 illustrates a proposed DP thymocyte–cTEC<sub>hi</sub> interaction and possible signalling mechanisms which may lead to [Ca<sup>2+</sup>] rises.

As illustrated in Figure 1.4, downstream of Lck and Syk activation, phosphorylation and activation of crucial linker/adaptor proteins by these kinases promotes formation of a membrane raft domain around the membrane
bound protein “linker for activation of T cells” (LAT; reviewed by Horejsi et al. 2010, Zhang et al. 1998). This macromolecular signalling complex provides scaffolding for the recruitment and activation of the enzymes PLC-γ1 (Zhu et al. 2003) and phosphoinositide 3 kinase (PI3K; Shim et al. 2011). Hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP2) by PLC-γ1 leads to a transient increase in the potent second messenger molecules inositol 1,4,5-trisphosphate (IP3) and membrane bound diacylglycerol (DAG). The former has the potential to bind to IP3 receptors (IP3R) on the ER to cause release of Ca2+ from stores (Streb et al. 1983). The latter can alter the local lipid composition in the PM or as a signalling molecule activate PKC (Szamel et al. 1989). The enzymatic activity of PI3K dynamically alters the concentration of PIP2 and phosphatidyl-inositol-3,4,5-trisphosphate (PIP3) within the local PM. Cellular imaging studies show that PIP2 and PIP3 localise to discrete microdomains in the PM. This restriction of lipid diffusion is thought to enable generation of local gradients, thereby enhancing the role of PIPs as regulatory molecules of PM proteins like ion channels (Hilgemann 2007, Wang & Richards 2012). Notably, dynamic changes in the local concentration of PIP2 and PIP3, and the generation of a broad range of endogenous lipid metabolites downstream of DAG, has been recognised as an important mechanism which can affect [Ca2+]i (reviewed in Ciardo & Ferrer-Montiel 2017, Cordero-Morales & Vásquez 2018, and Taberner et al. 2015).

How HS-enhanced autorosetting influences the DP thymocyte selection outcome remains a matter for further investigation. However, I note that a deleterious structural modification of the HS molecule (specifically in TECs) was found to increase the TCR repertoire of the peripheral CD8 T cell pool (Simon Davis, personal communication). In accordance with Grebe et al. (2004), this preliminary finding may suggest that in the absence of adequate TCR–MHC-I engagement, the role of this [Ca2+]i rise is perhaps to induce “death by instruction” in a subpopulation of preselection MHC-I restricted DP thymocytes. Facilitation of
rosetting by cTEC expressed HS binding to the thymocyte CD8 coreceptor might be required to promote a sustained stop signal in preselection thymocytes expressing poor affinity TCR.

Figure 1.4 A proposed HS priming mechanism involving CD8

Proposed mechanism for Ca\(^{2+}\) priming of the TCR signalling cascade during cTEC\(^{hi}\) (HS high) interaction. Without αβTCR–MHC engagement, HS binding of CD8 activates Lck signalling pathways and applies mechanical load on the PM which may be sufficient to transiently activate TCR associated stretch activated cation channels. Ca\(^{2+}\) influx, Lck activation of adaptor molecules including SLP-76 and cytoskeleton reorganisation leads to LFA1 conformational change and signalling. Synergistic Lck and LFA1 signalling promotes formation of the LAT signalsome and PLC-γ1 activation. PLC-γ1 hydrolysis of PIP\(_2\) leads to DAG production, PKC activation. Downstream of DAG production a non-selective cation channel promotes a sustained [Ca\(^{2+}\)]\(_i\) rise. ECM: extracellular matrix; Adaptor molecules: SLP-76:SH2 domain-containing leukocyte protein of 76 kDa; ADAP: adhesion and degranulation-promoting adaptor protein; WASP: Wiskott-Aldrich syndrome; Shc; Src homology domain containing. Cytoskeleton associated adaptor: talin1. Effector molecules: Lck, Syk: spleen tyrosine kinase; Itk: IL2-inducible T cell kinase; SHP-1: Src homology region 2 domain containing phosphatase; PKC: protein kinase C; stretch activated cation channel perhaps from TRP family or Piezo1.
1.3.1.7 **Sialylation of surface receptors modulates αβTCR reactivity**

As DP thymocytes continue to undergo positive selection and differentiation toward either CD4$^+$ or CD8$^+$ T cell lineage, there is associated upregulation of the TCR. In the CD8$^+$ population, this upregulation which has the potential to increase the reactivity to low affinity antigens, is counteracted by progressive glycosylation of the CD8 coreceptor (Daniels *et al.* 2001, Gil *et al.* 2008, Starr *et al.* 2003a). Physical modification of the CD8β structure by the addition of terminal sialic acid residues hinders CD8–MHC-I molecule interaction and hence impairs its role in stabilising weak affinity TCRs–pMHC-I interactions (Casabó *et al.* 1994, Li & Mariuzza 2013). Reducing the TCR–pMHC dwell time results in diminished signalling and provides a mechanism for tuning tolerance to self-peptides (Daniels *et al.* 2001). The added sialylation of CD8β has also been shown to inhibit HS binding to the coreceptor (Simon Davis 2015) and hence may act to negatively regulate rosette formation in positively selected thymocytes.

DP thymocytes that are positively selected continue to transition to become either CD4$^+$ or CD8$^+$ lineage single positive (SP) thymocytes. The lineage lines are restricted to binding either MHC-I or -II molecules, respectively (reviewed by Shah & Zúñiga-Pflücker 2014). During this period (~3 days; Jin *et al.* 2008b), the avidity of the αβTCR binding to the pMHC complex continues to be stringently tested. In addition, reactivity of the receptor to low affinity peptides decreases as sialylation of the surface receptors, in particular CD8, is progressively increased (Moody *et al.* 2001, Moody *et al.* 2003, Starr *et al.* 2003a). At the end of selection, it is critical that the repertoire of naïve CD4$^+$CD8$^+$TCR$^{hi}$ cytotoxic T cells, or CD4$^+$CD8$^+$ TCR$^{hi}$ helper T cells exiting the thymus has the potential to respond to foreign antigen presented on MHC molecules but shows tolerance and hence minimal reactivity to self-peptide-MHC engagement.
1.4 Ca\textsuperscript{2+} rises associated with thymocyte selection

In DP thymocytes, different patterns of [Ca\textsuperscript{2+}]i rises have been associated with different selection outcomes (Bhakta & Lewis 2005, Fu et al. 2009, Kane & Hedrick 1996, Kim et al. 2009a, Lo et al. 2012, Melichar et al. 2015, Ohno et al. 1991, Teh et al. 1997). This is supported by data presented in Figure 1.5.

1.4.1 Negative selection involves large amplitude [Ca\textsuperscript{2+}]i rises

In panel A (adapted from Fu et al. 2009), [Ca\textsuperscript{2+}]i responses were measured using flow cytometry in OT-1 transgenic (tg) thymocytes loaded with the Ca\textsuperscript{2+} indicator Indo-1, when stimulated with the peptide tetramers of known avidity to OT-1 tgTCRs. The strong response of the tgTCRs to the OVA peptide (epitope of ovalbumin) is associated with a large [Ca\textsuperscript{2+}]i rise (top) after exposure to normal extracellular Ca\textsuperscript{2+} at 2 min. This suggests that the rise in this case is largely carried by a transmembrane Ca\textsuperscript{2+} influx. Note that there is a much smaller rise immediately after the ova peptide is added to a nominally 0 mM Ca\textsuperscript{2+} solution, suggesting that with high affinity peptides, there is some contributing store release (see also below). The outcome of this type of Ca\textsuperscript{2+} rise was negative selection.

1.4.2 Positive selection involves a small, cumulative [Ca\textsuperscript{2+}]i increase

Variants of the ova epitope with less affinity to the OT-1 tgTCR are used from top to bottom with the top 4 resulting in negative and the bottom 5 in positive selection (Fu et al. 2009). Notably, in this in vitro study, the [Ca\textsuperscript{2+}]i rises associated with negative selection have an initial high peak amplitude, which declines slowly over minutes but remains well above resting [Ca\textsuperscript{2+}]. In contrast, when thymocytes were exposed to low avidity antigens (bottom 5 panels) in this reduced system, the positive selecting [Ca\textsuperscript{2+}]i rises increased slowly over time (5 min) and notably did not reach the amplitude triggered by high avidity antigens.
1.4.3  TCR–pMHC interaction duration reflects selection outcome

What is not captured in this figure is the much briefer duration of individual 
$[\text{Ca}^{2+}]_i$ rises that strongly correlate with periods of thymocyte migration arrest 
during which cell-cell interaction occurs (Bhakta & Lewis 2005). Figure 1.5B 
(adapted from Melichar et al. 2013) depicts $[\text{Ca}^{2+}]_i$ amplitude changes in 
individual DP thymocytes, detected using two-photon laser-scanning 
microscopy, as they migrated through a thymic slice, which likely represents a 
more biologically relevant environment. Not unexpectedly, $[\text{Ca}^{2+}]_i$ remained at 
resting level in HY$^{\text{CD4tg}}$ thymocytes (sensitive to an endogenous male peptide) 
seeded into a thymic slice from MHC I$^-$ mice, a mutation that limits thymocyte–
TEC interaction (non-selecting; Figure 1.5Bi). Without TCR–MHC interaction, 
these thymocytes will not receive survival signals and are predicted to die by 
neglect (Surh & Sprent 1994). When the same line of thymocytes was seeded onto 
a thymic slice derived from either a male (Figure 1.5Bii) or female mouse (Figure 
1.5Biii), the duration and amplitude of the resulting $[\text{Ca}^{2+}]_i$ rise were distinctly 
different. In the case of male peptides with high affinity to the TCR, the 
interaction with TEC (red line) were long lasting (>20 min) and the $[\text{Ca}^{2+}]_i$ was 
elevated throughout. During this time, summation of signalling responses 
triggered by repeated applied force transduced by TCR–pMHC–CD8 bond 
formation and disengagement induces enhanced $[\text{Ca}^{2+}]_i$ signalling (Pryshchep 
et al. 2014) which leads to negative selection. However, when exposed to female 
peptides (reduced affinity), the interactions were short lived (<5 min) with 
concomitant short rises above background $[\text{Ca}^{2+}]_i$ (dashed line).

1.4.4   A SOCE mechanism triggers negative selection

Aside from the differences in duration and amplitude, the mechanisms of the 
$\text{Ca}^{2+}$ rise driving negative selection are also distinctly different from that 
associated with positive selection (Fu et al. 2013). In the absence of extracellular
Ca$^{2+}$ (Figure 1.5A, top), negative selection is also associated with a rapid Ca$^{2+}$ rise of >550 nM (Nakayama et al. 1992) that includes ER store release (see also above). This suggests that a signalling cascade activates ER store release followed by store operated Ca$^{2+}$ entry (SOCE), resulting in a subsequent large Ca$^{2+}$ influx from the extracellular space. Certainly, in STIM deficient thymocytes, Oh-hora et al. (2013) found negative selection was reduced in favour of positive selection outcomes.

In mature T cells, activation of the “classical” SOCE channels (STIM1/Orai1) is considered the primary mechanism for Ca$^{2+}$ rise downstream of TCR activation (Feske et al. 2005). Highly selective for Ca$^{2+}$ ($P_{Ca}/P_{Na} > 1000:1$), functional STIM1/Orai channels are present in thymocytes, but the mechanisms which might differentially regulate their activity are poorly understood. In tumour derived Jurkat T cells (human CD4$^+$ leukemic T cells), the transient receptor potential vanilloid channel 2 (TRPV2) has been shown to positively modulate STIM1/Orai activity. Specifically, in cells transfected with a dominant-negative TRPV2 channel, Sauer and Jegla (2006) found that the previously observed SOCE downstream of TCR activation was absent in WT cells. Whether a similar mechanism is in place in thymocytes remains to be resolved.

1.4.5 **SOCE is not required for positive selection**

While large amplitude [Ca$^{2+}$]$_i$ rises coupled to SOCE play a central role in negative selection, this mechanism of “bulk” Ca$^{2+}$ entry is likely inappropriate for positive selection (Oh-hora et al. 2013). Rather, positive selection is triggered by repeated small amplitude [Ca$^{2+}$]$_i$ rises of 200 to 550 nM and an associated gradual elevation in background [Ca$^{2+}$]$_i$ over many hours (Nakayama et al. 1992, Ross et al. 2014). These transient rises do not appear to evoke detectable store release (Oh-hora et al. 2013), perhaps because signal transduction in positively selecting events is associated with delayed phosphorylation of LAT (Daniels et al. 2006).
The amplification of the signal may be insufficient to overcome the tight regulation of IP$_3$R activation by reversible tyrosine phosphorylation (Cui et al. 2004, Jayaraman et al. 1996) and binding of adaptor proteins, including Bcl-2 (Chen et al. 2004, Foyouzi-Youssefi et al. 2000, Rong et al. 2008, Rong et al. 2009, Zhong et al. 2006), thymocyte-expressed positive selection-associated 1 (Tespa 1; Wang et al. 2012) and thymocyte-expressed molecule involved in selection (Themis; Fu et al. 2013). The expression of anti-apoptotic proteins Bcl-2 and Bcl-X$_I$ is topographically regulated and their effect on thymocyte survival is likely to be complex. In DP thymocytes, a notable increase in Bcl-X$_I$ expression is proposed to down-regulate expression of the IP$_3$R and so modulate Ca$^{2+}$ homeostasis (Li et al. 2002a) and repress programmed cell death (Chao et al. 1995). While a 3-fold down-regulation of Bcl-2 is proposed to increase the susceptibility of DP thymocytes that fail positive selection, to programmed cell death. Intriguingly, within the DP population, a subset population (10–20%) scattered throughout the cortex has been shown to maintain high expression of Bcl-2 protein and these thymocytes are resistant to glucocorticoid induced death (Chao et al. 1995, Gratiot-Deans et al. 1993, Veis et al. 1993).

1.4.6 **TCR–MHC independent Ca$^{2+}$ rises**

Interestingly even in non-selecting thymic slice environments, sporadic Ca$^{2+}$ rises in DP thymocytes have been recorded (shown in Figure 1.5C; adapted from Bhakta et al. 2005). Occurring in approximately 15% of seeded thymocytes the authors proposed that these might be evoked by spontaneous integrin-mediated signal transduction. As mentioned previously, during thymocyte–TEC interaction and in the absence of TCR stimulation, CD8–MHC-I interaction alone caused apoptosis (Grebe et al. 2004). While the authors did not examine the [Ca$^{2+}$], other studies have reported that antibody cross-linking with an extracellular CD8 epitope lead to increased tyrosine phosphorylation of Lck (Irie et al. 1998,
Veillette et al. 1989). This, together with an increase in \([Ca^{2+}]_i\), perhaps via mechanical and/or Src kinase mediated activation of a \(Ca^{2+}\) permeable ion channel (Hisatsune et al. 2004, Jin et al. 2004, Vazquez et al. 2004, Xu et al. 2003), could provide the inside-out signal sufficient to induce a conformational change of LFA-1 to its receptive state. In conditions of suboptimal TCR stimulation, ligation of surface receptors such as CD2 and LFA-1 with their endogenous agonists present on the TECs has been shown to evoke \([Ca^{2+}]_i\) rises, most likely via synergistic enhancement of signal transduction and activation of different kinases, including the tyrosine kinases Lck, Syk and Fyn (Danielian et al. 1992, Kim et al. 2009a, Nonoyama et al. 1989, Paessens et al. 2008, Umehara et al. 1998, Walk et al. 1998, Zhao & Iwata 1995). In the case of preselection DP thymocyte–cTEC\(^{\text{hi}}\) interaction, activation of CD8 by HS binding (Simon Davis 2015) may further enhance signalling, potentially leading to activation of more than one \(Ca^{2+}\) mobilising mechanism and resulting in an enhanced \(Ca^{2+}\) rise, as illustrated in Figure 1.4. With regard to the current research, the \([Ca^{2+}]_i\) rise induced by the addition of DxS is thought to be enabled by activation of such a synergistic signalling pathway.
Figure 1.5 Selection associated Ca\textsuperscript{2+} fluxes in DP thymocytes

(A) Ca\textsuperscript{2+} time courses measured using flow cytometry in OT-1 thymocytes responding to the addition of antigen peptides OVA–Catnb (from top to bottom). In all samples, the [Ca\textsuperscript{2+}]\textsubscript{o} was initially kept nominally 0 mM and was restored to normal after 2 min. Note the change in scale of the abscissa from top to bottom. (B) Plots of Ca\textsuperscript{2+} ratio in individual thymocytes under (i) non-selecting, negative (ii) and positive (iii) conditions. The red line indicates period of thymocyte-TEC interaction. Dashed line indicates the upper limit of background [Ca\textsuperscript{2+}]\textsubscript{i} under resting conditions. (C) Spontaneous [Ca\textsuperscript{2+}]\textsubscript{i} rise occurring during a non-selecting condition.
1.5 The “Tellam-Parish” Ca\(^{2+}\) rise

Central to this thesis is the study by Tellam and Parish (1987) who described a sustained [Ca\(^{2+}\)]\(_i\) rise that was induced in thymocytes following the addition of 0.1 mg/mL Dxs, but not by the addition of other smaller mol. wt. polysulfated polysaccharides. The rise was carried by an influx of Ca\(^{2+}\) across the PM and seemed independent of ER store release as EGTA chelation of [Ca\(^{2+}\)]\(_o\) abolished it. Subsequent investigations found this Dxs induced [Ca\(^{2+}\)]\(_i\) rise was specific to glucocorticoid-sensitive DP thymocytes (Weston et al. 1991), indicative of preselection DP thymocytes (Ohoka et al. 1996). As shown in Figure 1.3D, Dxs added to a thymocytes in a spectroscopy cuvette resulted in a biphasic [Ca\(^{2+}\)]\(_i\) rise. This rise comprised a small increase shortly after the addition of Dxs followed approximately 4 min later by a significantly larger [Ca\(^{2+}\)]\(_i\) rise of 165 ± 11 nM (Tellam & Parish 1987), which slowly reached a sustained maximum after 15 min.

However, distinct from Dxs, the addition of 0.1 mg/mL heparin (12.5 kDa) or 0.1 mg/mL Dxs (5 kDa) to DP thymocytes in suspension elicited only a negligible [Ca\(^{2+}\)]\(_i\) increase (Tellam & Parish 1987). This observation suggests that activation of other surface receptors and adhesion molecules may be involved in causing the [Ca\(^{2+}\)]\(_i\) rise. It appears that in contrast to heparin and low mol. wt. Dxs (5 kDa), the significantly larger Dxs molecule interacts not only with CD8\(β\) but potentially also binds and/or cross-links with other surface receptors.

1.6 Overview of Ca\(^{2+}\)-handling mechanisms in thymocytes

As proposed earlier, to modulate the resting [Ca\(^{2+}\)]\(_i\) and shape Ca\(^{2+}\) rises downstream of thymocyte–TEC interaction there must be a range of Ca\(^{2+}\) handling mechanisms in place (Badou et al. 2013, Feske 2013, Matza et al. 2016). In vivo, multiple signalling receptors may be activated during thymocyte–TEC interaction, leading to synergistic enhancement of signal transduction via
activation of different kinases. These include the tyrosine kinases Lck, Syk and Fyn (Danielian et al. 1992, Nonoyama et al. 1989, Paessens et al. 2008, Umehara et al. 1998, Walk et al. 1998, Zhao & Iwata 1995), protein kinase C (PKC) isoforms and the phosphatase CD45, which together potentially result in the activation of more than a single Ca\(^{2+}\) mobilising target (reviewed by Kotturi et al. 2006, Shiroo et al. 1992).

An increasing number of ion channels and transporters some of which are Ca\(^{2+}\) permeable, have been positively identified in thymocytes though, often, their functional role remains unclear. While different patterns of Ca\(^{2+}\) rises are required during thymopoiesis, knowledge of the molecular nature of the respective Ca\(^{2+}\) rises involved in positive selection, negative selection and in the mechanism underpinning death by neglect or “death by instruction” (Grebe et al. 2004) remains incomplete. In the next sections, I will review some of the ion channels and transporters proposed to have roles in thymopoiesis and also channels, not specifically identified in thymocytes, but considered as potential candidates in the Dxs [Ca\(^{2+}\)].

1.6.1 Ion channels and transporters identified in thymocytes

Table 1.1 lists functional Ca\(^{2+}\) permeable channels, ion transporters/exchangers and Na\(^+\), K\(^+\) and Cl\(^-\)-selective channels reportedly present in thymocytes that potentially affect [Ca\(^{2+}\)]; homeostasis and/or mediate distinct [Ca\(^{2+}\)]; signals.

1.6.1.1 Voltage-gated cation channels

Included are several channels that activate in response to local changes in PM potential, and as indicated by their name, these voltage-gated channels, Kv, Cav and Nav are highly selective for K\(^+\), Ca\(^{2+}\) and Na\(^+\), respectively. Upon reaching a threshold voltage these channels undergo a rapid conformational change that opens the pore and allows the flow of ions down the electrochemical gradient.
As in excitable cells, Kv channels have a role in restoring the resting membrane potential ($V_m$) following depolarisation and so can modulate the influx of both Ca$^{2+}$ and Na$^+$ through other open channels. However, it is notable that prolonged or repeated depolarisation will in activate Kv1.3 (reviewed by Cahalan et al. 2001).

Cav channels have been well described in excitable cells (Hille 2001). While a few voltage-dependent Ca$^{2+}$ channels (typically T- and some L-type) can be activated at low voltage that is not far from the resting membrane voltage which is typically around -60 mV, most Cav channels (N-, P/Q- R- and most L-type) require considerable membrane depolarisation (typically ≥ -25 mV) to stimulate their gating. However, in lymphocytes this dependency on voltage-gating may be lost. Recent evidence suggests that the L-type channels expressed in T lymphocytes are structurally distinct and may be insensitive to membrane depolarization. As a consequence of alternative pre-mRNA splicing, Cav1.1 and Cav1.4 channels are made up of subunits with a disrupted “voltage sensing” domain. Consequently, these channels are not significantly gated by membrane depolarisation (reviewed by Davenport et al. 2015, Kotturi & Jefferies 2005, Matza et al. 2016). Rather, constitutive activity of these channels is thought to be inhibited by binding of the calcium sensing molecule STIM 1 at the channel C-terminal region (Park et al. 2010, Wang et al. 2010, Zinchenko et al. 2009).
### Table 1.1 Ion channels and transporters detected in thymocytes

<table>
<thead>
<tr>
<th>Channel type</th>
<th>gene</th>
<th>Ion permeability</th>
<th>Source(s)</th>
</tr>
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<tbody>
<tr>
<td>Nav</td>
<td>Scn4b</td>
<td>Na⁺</td>
<td>(Lo et al. 2012)</td>
</tr>
<tr>
<td></td>
<td>Scn5a (Nav1.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KᵥKa</td>
<td>Kcnm4 (Kᵥ3.1)</td>
<td>K⁺</td>
<td>(Mahaut-Smith &amp; Mason 1991)</td>
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<td>Kcn1 (Kᵥ1.1)</td>
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<td>CIC</td>
<td>Clcn2</td>
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<td>Cav</td>
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<td>Cacna1f (Cav1.4)</td>
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<td>NSC</td>
<td>(Jin et al. 2008a)</td>
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<td>NSC</td>
<td>(Gasser &amp; Guse 2005)</td>
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<td>NSC</td>
<td>(indirect; Hurne et al. 2002, Koh et al. 1998)</td>
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<td></td>
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<td>NSC</td>
<td>(Amantini et al. 2004, Farfariello et al. 2012)</td>
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<td>(Echevarria-Lima et al. 2003)</td>
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1.6.1.2 **Voltage-independent Ca²⁺ permeable channels**

Also listed in Table 1.1 are the voltage-independent cyclic nucleotide gated (CNG) channels and transient receptor potential (TRP) channel family. Typically, these channels do not require a depolarisation for activation, although some TRP channels display non-classical voltage sensitivity (reviewed by Bertin & Raz 2016). Instead, their activation may be due to locally experienced factors like the metabolic state, availability of 2nd messengers, temperature and mechanical load. Distinct from Cav channels, TRP and CNG are typically cation-selective and consequently, may have only a partial conductance for Ca²⁺ (Cahalan & Chandy 2009, Ramsey et al. 2006, Robert et al. 2011, Varnum & Dai 2015).

1.6.1.3 **Ligand-gated Ca²⁺ permeable channels**

Other non-selective cation channels reportedly expressed in thymocytes are the ionotropic glutamatergic (iGluR), purinergic (P2XR) and nicotinic acetylcholine (nAChR) receptors. Activation of these channels depends upon agonist ligand (glutamate, adenosine triphosphate and acetylcholine, respectively) binding at the receptor site, to induce a conformation change in the protein that gates the pore (Burnstock & Boeynaems 2014, Fujii et al. 2017).

1.6.1.4 **2nd messenger-gated channels**

In thymocytes the generation of second messenger signalling molecules, including IP₃, DAG and Ca²⁺ facilities activation of 2nd messenger-gated channels expressed on the ER/mitochondrial membrane as well as the PM. IP₃ triggered activation of IP₃R located in the ER membrane and Ca²⁺ mediated ryanodine receptor activation results in ER Ca²⁺ release and subsequent activation of SOCE channels, most notably Orai channels. Debatably, some canonical TRP (TRPC) and vanilloid TRP (TRPV) channels also function as SOCE channels (DeHaven et al. 2009). While the STIM/Orai channel complex and TRPV6 are highly selective
for Ca^{2+}, TRPC channels are non-selective cation channels with variable selectively for divalent vs. monovalent cations (reviewed by Gees et al. 2011). Since TRP channels are thought to be involved in the DxS [Ca^{2+}], they are reviewed in more detail in section 1.7.2.

1.6.1.5 Ca^{2+} activated K+ channels

Thought to be important in enabling sustained Ca^{2+} entry through activated SOCE channels in T-lymphocytes (reviewed by Chandy et al. 2004), the Ca^{2+} activated K+ channel K_{Ca}3.1 is also expressed by thymocytes. Located on the PM, channel gating is regulated by the Ca^{2+} sensing protein calmodulin which is tightly bound in the K_{Ca}3.1 C-terminal region. Co-located with Ca^{2+} permeable channels, K_{Ca}3.1 activation is triggered when a rise in [Ca^{2+}]_i results in Ca^{2+}–calmodulin binding (K_d ~300 nM; reviewed by Cahalan et al. 2001). The consequent K+ efflux via this intermediate conductance channel induces a repolarization, effectively lowering V_m and so increasing the electrical driving force for Ca^{2+} to enter the cell.

1.6.2 Crucial channels in thymopoiesis

Our understanding of channels crucial to thymocyte maturation is far from complete. Ca^{2+} signalling during thymopoiesis is likely to be complex, highly variable and dependent upon orchestration by multiple channels that may well be differentially expressed at distinct stages of development and in response to different signalling pathways. Of the channels listed in Table 1.1, a few have been identified as having a critical role in thymopoiesis (see sections below). In general, mutations affecting the function of these channels has been shown to alter the outcome of thymocyte maturation, e.g. reducing the size and/or altering the ratio of CD8+ and CD4+ lineage cells in the peripheral T-cell population.
1.6.2.1 TRPM7\(^{-}\) causes impaired DN3 differentiation

Notably, deletion of the transient receptor potential melastatin 7 (TRPM7) channel results in a partial block of thymocyte maturation beyond the DN3 phase and significant reduction of the DP and CD4 SP populations (Jin et al. 2008a). TRPM7 forms an unusual channel–kinase complex (discussed further in 1.7.3.3.4) and is proposed to play a role in Mg\(^{2+}\) homeostasis. However, in the TRPM7 deficient thymocytes cellular Mg\(^{2+}\) was not apparently disturbed. Rather, these authors observed a change in thymic architecture and mTEC maintenance. As development of the varied thymic microenvironments is crucially influenced by thymocyte–TEC cross-talk, perhaps as observed in developing lung fibroblasts (Wei et al. 2009), a loss of TRPM7-evoked “Ca\(^{2+}\) flickers” similarly impairs thymocyte migration and adhesion. Typical of TRP family channels, TRPM7 is gated by multimodal mechanisms, including direct activation by membrane stretch (Numata et al. 2007). It is well established (reviewed by Takahama 2006) that thymocyte–stromal cell interactions across varied thymic microenvironments are pivotal to thymocyte maturation. Failure to receive the necessary development signals from stromal cells will disrupt thymocyte proliferation and differentiation (reviewed by Dzhagalov & Phee 2012, van Ewijk et al. 2000, van Ewijk et al. 1994).

1.6.2.2 Kv1.1 and 1.3 channels indirectly modulate Ca\(^{2+}\) entry

While not permeable to Ca\(^{2+}\), the voltage-gated K\(^{+}\) channels, Kv1.1 and Kv1.3 also play a role in early thymopoiesis and are proposed to indirectly modulate Ca\(^{2+}\) entry. Specifically, Freedman et al. (1995) showed the addition of dentrotoxin and charybdotoxin (Kv1.1 and Kv1.3 antagonists, respectively) significantly reduced DN thymocyte proliferation and decreased the DP population in fetal thymic organ cultures. In T lymphocytes, Kv1.3 channels activate at >-50 \(V_m\) and reach half maximal activation at ~-30 mV (Hajdu et al. 2003, McKinnon & Ceredig 1986).
As $V_m$ in T lymphocytes is maintained between -50 to -70 mV (reviewed by Cahalan & Chandy 2009, Ishida & Chused 1993) it is likely a small number Kv1.3 channels are readily activated. Comparable to the $V_m$ value (-50 mV) proposed by Cahalan and Chandy (2009), I found the resting $V_m$ in thymocytes to be 49.5 ± 12.5 mV, $n = 15$. This value was obtained from patch clamp experiments conducted as part of my honours research project in 2012 (Feakes 2012). Not unexpectedly, in T lymphocytes, inhibition of these K+ channels, which normally maintain the electronegative resting membrane potential by efflux of K+ (Hess et al. 1993), causes membrane depolarisation which consequently reduces Ca$^{2+}$ influx (Hess et al. 1993, Liu et al. 2002).

Surprisingly however, thymocyte proliferation and development is normal in Kv1.3$^{-/-}$ mice. In the Kv1.3 deficient thymocytes, Koni et al. (2003) found Cl$^-$ conductance was increased by 50-fold which, they suggest, restored the normal $V_m$. This compensatory mechanism indirectly supports the role of Kv channels in maintaining normal $V_m$, a requirement for normal Ca$^{2+}$ influx (Hess et al. 1993) and DN thymocyte proliferation and differentiation.

1.6.2.3 Cav(β2)$^{-/-}$ causes impaired DN differentiation

Disrupted differentiation from the DN3 to DN4 stage has been reported in thymocyte precursors deficient in the β2 regulatory subunit of voltage-gated Ca$^{2+}$ channels (Cavβ2; Jha et al. 2015). In DN thymocytes it appears the β2 regulatory subunit combines with the 1.2 or 1.3 isoform of the Cav pore forming α subunit to create functional channels. Consequently, the abundance of Cav1.2 and Cav1.3 channels was found to be reduced in Cavβ2 deficient thymocytes. Examining the effect on thymopoiesis, Jha et al. (2015) found the DP population and consequently the peripheral T cell pool were substantially depleted. This perhaps reflects an alteration of the crucial biphasic Ca$^{2+}$ signals associated with β-selection. Whether these channels continue to have roles in DP selection
processes and/or death by instruction/neglect is not known particularly as expression of the α and β subunits of Cav varies markedly between DN, DP and SP thymocytes and T lymphocytes (Badou et al. 2013, Badou et al. 2006, Jha et al. 2015, Matza et al. 2016, Omilusik et al. 2011).

1.6.2.4 Cav1.4 and Nav1.5 regulate CD4 lineage selection

L-type Cav1.4 (Omilusik et al. 2011) and the TTX insensitive voltage-gated Na⁺ channel Nav 1.5 (Scn5a⁺/− and Scn4β⁺/−; Lo et al. 2012) have been shown to be important for lineage dependent differentiation in thymopoiesis. Analysis of the SP CD4 and SP CD8 in thymocytes deficient in either of these channels showed a significant decrease in the number of SP CD4 cells, while the SP CD8 population was not markedly affected. In these thymocytes, TCR activated Ca²⁺ signalling was shown to be significantly reduced however, SP CD8 differentiation again appeared relatively unchanged. Interestingly, McRory et al. (2004) found very few cells in the thymus positively stained for Cav1.4 protein. This might indicate that, rather than being expressed by DP thymocytes, Cav1.4 channel activity may be required at a later developmental stage where it crucially regulates CD4 lineage differentiation.

1.6.2.5 Orai/STIM channels are not critical for thymocyte maturation

While the SOCE channels STIM/Orai (Feske et al. 2005) and Cav1 channels (particularly Cav1.4 channels which contain a unique α4S and β4 subunits) have been shown to play crucial roles in mature T cell survival and function, thymocytes deficient in these genes show only subtle changes in development (Kotturi & Jefferies 2005, Oh-hora et al. 2013, Omilusik et al. 2011). Perhaps this reflects different and limited expression of these channels in the thymocyte PM compared to naïve and mature T cells (Badou et al. 2006, Kotturi & Jefferies 2005, McRory et al. 2004).
The expression profile of Orai and Stim isoforms vary at distinct stages of T cell maturation (as shown in Fig. 1.6, an extract from the BioGPS database; Barrett et al. 2013, Edgar et al. 2002). This phenomenon may reflect the apparent difference in dependence on STIM/Orai channels for normal function. In the BioGPS database the median value (M) is derived from the gene expression profile from an array of normal mouse embryonic and mature tissues, cell lines and organs. In each case, the gene expression profile is shown relative to the median value which was determined from the expression profile from a diverse range (96) of normal tissues, organs, and cell lines obtained from naïve male C57BL/6 mice and hybridized to MOE_2 arrays.

Notably, in thymocytes the expression profile of Stim2 is higher than that of Stim1 (Figure 1.6B). An increase in STIM2 protein perhaps also provides an additional mechanism for inhibition of Orai channel Ca\textsuperscript{2+} influx during early T cell development. What cannot be deduced from the Bio GPS data and remains to be investigated, is the relative expression in thymocytes of the Stim2 splice variants: Stim2α and Stim2β. Distinct from STIM1 and STIM2α, STIM2β has been shown to block Orai channel activation and hence negatively modulate SOCE (Rana et al. 2015).

Related to Orai channel activation, IP\textsubscript{3}R-mediated Ca\textsuperscript{2+} release has also been shown to play crucial role in directing thymopoiesis and programmed cell death (Khan et al. 1996, Ouyang et al. 2014). However, the significant redundancy within the IP\textsubscript{3}R genes (Itpr1, Itpr2 & Itpr3) ensures normal DP thymocyte development; such that, Ouyang et al. (2014) found transition from the DN4 to DP stage was significantly disrupted only when all 3 genes were conditionally knocked out.

Aside from the well-described role the IP\textsubscript{3}Rs play in the release of Ca\textsuperscript{2+} from the ER (reviewed by Fedorenko et al. 2014), in thymocytes ~30% of IP\textsubscript{3}Rs are enriched
with sialic acid and proposed to be located at the PM. Distinct from the ER located receptor, the PM associated IP$_3$R has a higher affinity for inositol 1,3,4,5-tetrakisphosphate (IP$_4$) (Khan et al. 1992b). Proposed roles for these PM bound IP$_3$Rs are facilitation of the [Ca$^{2+}$]$_i$ plateau phase observed in lymphocytes after a proliferative stimulus (Khan et al. 1992a) and regulation of apoptosis in thymocytes via modulation of capacitive Ca$^{2+}$ influx (Khan et al. 1996).

**Figure 1.6 Relative Orai expression profile in T lymphocyte populations**

mRNA expression data detailed in the BioGPS database (dataset: GeneAtlas MOE430, gcrma) which shows the variation in expression of *Orai* and *Stim* isotopes during T cell maturation. A. (i) *Orai1*: probe set 1424990_at; (ii) *Orai2*: probe set 1424990_at; (iii) *Orai3*: probe set 1424990_at. B. (i) *Stim1*: probe set 1436945_x_at; (ii) *Stim2*: probe set 1441024_at.
Channels linked to thymocyte apoptosis

Channels reported as having a role in thymocyte death, have in general been determined through the application of either glucocorticoids or adenosine 5’-triphosphate (ATP; or its analogue). Studying the effect of 0.1 µM dexamethasone on rat thymocytes, Khan et al. (1996) noted a 20-fold increase in IP₃R (type 3) protein at the PM, an associated Ca²⁺ rise and induced apoptotic changes. Significantly, in thymocytes transfected with antisense IP₃R3 mRNA the dexamethasone evoked changes were abolished. Based on these findings the authors propose that that the PM IP₃R3 provided a mechanism for Ca²⁺ entry that lead to cell apoptosis. Notably, their results do not rule out the possibility that another channel was responsible for the Ca²⁺ flux.

For instance, canonical transient receptor potential channels (TRPC; further reviewed in 1.7.3) contain regulatory IP₃R/calmodulin (CaM) binding site within their C-terminal region (Kiselyov et al. 1998, Kwon et al. 2007, Tang et al. 2001, Yuan et al. 2003). While the expression of TRPC channels in thymocytes remains to be determined, they have been reported in other T lymphocytes and so may provide an IP₃R-dependent mechanism for Ca²⁺ influx (Kim et al. 2006, Vazquez et al. 2006). While the involvement of TRPC channels in apoptotic signalling pathways remains undefined, it is noted that, TRPC3 and TRPC6 activation have been linked to apoptosis in septic T-lymphocytes.

1.6.3.1 P2X receptors

While purinoreceptors (P2XRs) do not play a role in glucocorticoid mediated thymocyte death (Jiang et al. 1996), their ligand activation by extracellular ATP has been linked to thymocyte apoptosis. P2X1, -X2, -X6 and -X7 channels are reportedly expressed in thymocytes (see Table 1.1). Located in the PM, these ligand-gated channels are rapidly activated by extracellular ATP and commonly
are permeable to Ca\(^{2+}\), Na\(^+\) and K\(^+\). In thymocytes, apoptosis can be induced upon activation of P2X7R (Courageot et al. 2004, Le Stunff et al. 2004) and perhaps P2X1R (Ross et al. 1997). Investigating the mechanism involved, Shoji et al. (2014) found in mature murine T cells, non-selective pannexin1 channels (Panx1) modulated the Ca\(^{2+}\) influx observed in response to P2X7R activation. But notably, in T cells obtained from Panx1\(^{-/-}\) mice, the ATP induced Ca\(^{2+}\) was not abolished. While this functional role was studied using mature T cells, Locovei et al. (2007) have provided evidence of functional Panx1 channels in thymocytes. These large pore forming channels are described as having variable conductance, that ranges from 50–500 pS depending upon the activating stimuli. In their high conductance state Panx1 channels may allow ATP efflux which in turn provides a feedback stimulus that acts on the P2XR and promotes Ca\(^{2+}\) waves (reviewed by Whyte-Fagundes & Zoidl 2018).

Adding further complexity to the ATP activation of thymocyte purinoreceptors and the resultant Ca\(^{2+}\)-driven apoptotic response (reviews by Alves et al. 1999, and Burnstock & Boeynaems 2014) the mechanism may also be specific to the stage of thymocyte development (El-Moatassim et al. 1989, Ross et al. 1997).

1.6.4 Transporters, exchangers and channels that can modulate [Ca\(^{2+}\)].

As mentioned earlier, [Ca\(^{2+}\)]\(_{i}\) rises associated with activation of Ca\(^{2+}\) permeable channels, are countered by the activation of ion transporters which may be facilitated by the transmembrane flux of ions besides Ca\(^{2+}\) via Na\(^+\), K\(^+\) or Cl\(^-\) channels. Figure 1.7 schematically illustrates how the interplay between channels and transporter activity might modulate [Ca\(^{2+}\)]\(_{i}\). Active ion transport is facilitated by Ca\(^{2+}\)-ATPase pumps on the PM and ER (PMCA and SERCA, respectively). Deriving energy from ATP hydrolysis, these pumps remove Ca\(^{2+}\) from the cytosol into the extracellular milieu or the ER lumen by transporting Ca\(^{2+}\) against its concentration gradient.
In thymocytes, the moderate constitutive leak of Ca\(^{2+}\) from the ER is normally balanced by Ca\(^{2+}\) reuptake via the SERCA pump. Pharmacological inhibition of SERCA, using drugs such as cyclopiazonic acid (CPA) or thapsigargin, provides evidence of the leak and is a commonly used method to induce depletion of [Ca\(^{2+}\)]\(_{ER}\). This protocol enables investigation of the relationship between the ER store release and various Ca\(^{2+}\) entry mechanisms via channels or transporters.

Transport of Ca\(^{2+}\) is commonly enabled by Na\(^+\)/Ca\(^{2+}\) antiporters, which are also referred to as exchangers. Of the 3 genes encoding sodium-calcium exchangers (Slc\(\text{ca8a1–slc\(\text{ca8a3}\)}\)), Slc\(\text{ca8a1}\) (NCX1) has the highest expression in DP thymocytes (>3 x median expression; probeset:1420210_at; dataset: GeneAtlas MOE430, gcrma; BioGPS data base). NCX uses the concentration gradients of Ca\(^{2+}\) and Na\(^{+}\) to couple transport (in opposing directions) of 1 Ca\(^{2+}\) against 3 Na\(^{+}\) producing a net ion flux that is electrogenic. Importantly, the direction of the ion exchange is not fixed and NCX activity may shift between “forward” and “reverse” modes depending upon ion concentrations and V\(_m\). The extrusion of Ca\(^{2+}\) and influx of Na\(^{+}\) during forward (exit) mode causes a depolarization, while the reverse (entry) mode which brings Ca\(^{2+}\) into the cytosol and extrudes Na\(^{+}\) results in a hyperpolarization. Having a markedly higher maximal rate than the PMCA, activation of the NCX can rapidly alter V\(_m\), thereby altering the electrochemical driving force and affecting the [Ca\(^{2+}\)] and also [Na\(^{+}\)]. (reviewed by Blaustein & Lederer 1999, Verkhratsky et al. 2017).

In activated human peripheral T cells and Jurkat cells, Wacholtz et al. (1993) found that pharmacological block of NCX activity reduced the movement of Ca\(^{2+}\) across the PM and so reduced the expected TCR-induced increase in [Ca\(^{2+}\)]. Based on their findings they proposed that in T lymphocytes reverse mode NCX activity may sustain a [Ca\(^{2+}\)] rise. However contrary to their results, Donnadieu
and Trautmann (1993) found no evidence to support reverse mode NCX activity in these cells.

In murine thymocytes evidence for functional NCX activity is reported by Echevarria-Lima et al. (2003). Their research demonstrated reverse mode activation of the NCX, when the Na\(^+\) gradient was pharmacologically lowered by the addition of ouabain (Echevarria-Lima et al. 2003), which blocks the sodium-potassium ATPase (Na-K-ATPase; preventing K\(^+\) influx and Na\(^+\) extrusion at a ratio of 2:3). However, activation of this mode under physiological conditions remains to be determined. The use of drugs such as YM244769 and SN-6, which preferentially inhibit the NCX reverse mode, may provide more information on a role for NCX in facilitation of sustained [Ca\(^{2+}\)]\(_i\) rises.

Additional to NCX, sodium-potassium-calcium exchangers (NCKX) co-transport Ca\(^{2+}\) and K\(^+\) across the PM using energy derived from the ion gradients of Na\(^+\) and to some extent K\(^+\). In DP thymocytes, expression of the genes encoding NCKX exchangers (Slc24a1-Slc24a4) does not exceed the median value and a functional role for NCKX in thymocytes is uncertain. The transport of 4 Na\(^+\) counter to 1 Ca\(^{2+}\) plus 1 K\(^+\) results in the net movement of one positive charge and like the NCX, NCKX activity also produces a change in \(V_m\) (reviewed in Altimimi & Schnetkamp 2007).

The local electrogenesis of such transporters can alter \(V_m\), and consequently vary the transmembrane ionic driving force acting on Ca\(^{2+}\). Responsible for setting the [Na\(^+\)]\(_i\), the Na\(^+\)-K\(^+\)-ATPase actively exchanges 3 cytosolic Na\(^+\) ions for 2 extracellular K\(^+\) ions. This acts to lower \(V_m\) and maintain the gradient for Na\(^+\) and also Ca\(^{2+}\) to enter the cell (Skou & Esmann 1992). In T lymphocytes, Ishida and Chused (1993) found inhibition of the Na\(^+\)-K\(^+\)-ATPase with ouabain, or removal of [K\(^-\)]\(_o\), caused a depolarisation of T lymphocytes. Similarly, margatoxin or charybdotoxin induced inhibition of Kv channels in lymphocytes causes a depolarization
and attenuates the amplitudes of $[\text{Ca}^{2+}]_i$ rises necessary for T cell activation and DP thymocyte differentiation (Koo et al. 1997, Leonard et al. 1992).

Additional to these transporters, there are Ca$^{2+}$ transporters located on the inner mitochondrial membrane. Importantly, these transporters can modify oscillations in $[\text{Ca}^{2+}]_i$. Activation of the mitochondrial Ca$^{2+}$ uniporter (MCU), Na$^+$-Ca$^{2+}$ exchangers (NCLX) and Ca$^{2+}$-H$^+$ antiporter facilitates the sequestration and release of Ca$^{2+}$ into and from mitochondria. This mitochondrial “buffering” of $[\text{Ca}^{2+}]_i$ rises enables the slow tuning their amplitudes and decays (Santo-Domingo & Demaurex 2010).

Figure 1.7 Mechanisms involved in [Ca$^{2+}$] homeostasis

Schematic drawing of movement of ions via a broad range of ion channels, SERCA and PMCA Ca$^{2+}$ pumps, NCXs (working in forward or reverse mode), mitochondrial Ca$^{2+}$ uniporter (MCU) voltage-dependent anion channel and sodium-hydrogen exchanger (NHE). The interplay of these ionic mechanisms enables the removal of cytosolic Ca$^{2+}$ against the concentration gradient into the ER, mitochondria and extracellular space. The movement of monovalent ions (Na$^+$, K$^+$, H$^+$ and Cl$^-$) across the various membranes via activated channels and or transporters locally alters the membrane voltage potential and modulates the driving force acting on Ca$^{2+}$ influx.
1.6.4.1 Regulation of channels and transporters by membrane lipids


In many cell types including T lymphocytes, rapid phosphorylation of DAG by diacylglycerol kinases is thought to curb initial DAG induced signalling. However, subsequent activation of PKC and promotion of phospholipase D (PLD) and phosphatidic acid (PA) can result in a second and more sustained increase in DAG and long lasting activation of PKC (reviewed by Nishizuka 1992, Outram et al. 2002, Szamel et al. 1989).
Beyond this, subsequent hydrolysis of DAG by DAG lipase and parallel cytosolic phospholipase A₂ (cPLA₂) enzymatic activity leads to the production of AA, a major regulatory lipid which can also have signalling properties. In turn, oxidization of AA via either the lipoxygenase, cyclooxygenase or cytochrome-P450 metabolic pathway gives rise to a multitude of eicosanoids (Rousseau et al. 2015) which also have potent signalling properties. Figure 1.8A schematically illustrates how the generation of the lipid signalling molecules DAG and AA downstream of activation of PLC-γ1, PKC, PLD₃ and PLA₂ and how the relative increase of these lipids within the cell over time (Fig1.8B) may lead in activation of DAG and AA sensitive TRP channels and sustained Ca²⁺ entry (adapted from Nishizuka 1992, and Szamel et al. 1989).

Figure 1.8 DAG and AA generation downstream of PLC activation

A. Schematic overview of how production of DAG and AA downstream of PIP₂ hydrolysis by PLC-γ₁, and phosphatidylcholine (PC) hydrolysis by phospholipase D₃ (PLD₃) and PLA₂ may promote activation of TRP channels leading to Ca²⁺ entry. Downstream of PKC activation by DAG, activation of PLD₃ promotes ongoing DAG production via phosphatidic acid (PA). Production of free fatty acids (FFA), notably AA, by activated PLA₂ may further modulate TRP channel activity and Ca²⁺ influx. Rises in [Ca²⁺]ᵢ can influence various cellular responses. B. Provides a schematic time course of DAG (pink) and AA (red) production over time. The first distinct rise(1) in DAG is in response to activation of PLC-γ₁ (yellow arrow), while a second rise (2) occurs following PLD₃ activation. Shown in (A) PLD₃ and PLA₂ activation occurs following production of DAG and Ca²⁺ dependent PKC activation and subsequent hydrolysis of PC leads to increased AA production over time. Adapted from Nishizuka (1992).
Not surprisingly, many of these lipid mediators have been proposed as direct and indirect regulators of ion channel gating, in particular channels belonging to the large TRP family (reviewed in Bang et al. 2012, Caires et al. 2017, Ciardo & Ferrer-Montiel 2017, Eijkelkamp et al. 2013, Hwang et al. 2000, Sisignano et al. 2014, Taberner et al. 2015, Vazquez et al. 2010) and also Piezo channels (Borbiro et al. 2015).

1.6.4.1.1 Modulation of ion channels by GAGs including DxS

For this thesis, DxS was commonly used to evoke the specific Ca\(^{2+}\) rise in DP thymocytes (described in Section 1.5). While direct channel gating by DxS is not considered the primary mechanism for initiating this Ca\(^{2+}\) entry, it is noted that HS and other large sulfated polysaccharides, including DxS, can directly modify the activity of some ion channels. Specifically, both HS and DxS have been found to affect AMPA receptors in rat hippocampal neurons. Specifically, direct polysaccharide–AMPA receptor interaction was shown to enhance channel opening time and subsequently potentiate Ca\(^{2+}\) entry (Chicoine & Bahr 2007, Chicoine et al. 2004, Suppiramaniam et al. 2006). Additionally, externally applied heparin has an agonistic effect on Cav1 channels (in rabbit skeletal muscle cells; Knaus et al. 1992, Knaus et al. 1990). While AMPA receptors are reportedly involved in integrin-mediated adhesion in T cells (Ganor et al. 2003), their presence in DP thymocytes has not been established. However, L-type Ca\(^{2+}\) channels have been identified on thymocytes (Omilusik et al. 2011) and so perhaps might be potential binding targets of GAGs.

1.7 Overview of channels that may enable the DxS [Ca\(^{2+}\)]\(_i\) rise

Investigating the DxS [Ca\(^{2+}\)]\(_i\) rise, Tellam and Parish (1987) showed the Ca\(^{2+}\) influx was not altered by membrane depolarisation (induced by increasing [K\(^+\)]\(_o\) to 60 mM) or by the addition of 3 µM nifedipine (a Cav1 type channel antagonist).
Additional to these results, pharmacological blocking experiments undertaken in 2012 (as my honours research project; outlined in Appendix 3; p.353) rule out Cav2 and 3 type voltage-gated Ca\(^{2+}\) channels, ligand gated receptors (specifically the P2XR, iGluR, PM bound IP\(_3\)R and nAChR) and the cyclic nucleotide gated channel CNGA1 as likely candidates. Rather, a Piezo channel and/or channels from the TRP family were considered potential candidates, in particular channels proposed to be mechanosensitive, modulated by Src kinase and membrane lipids.

1.7.1 **Piezo channels**

The recently identified Piezo channel family is small, having two members identified as Piezo1 and Piezo 2 (Coste et al. 2010). Comprising >2500 amino acid residues these proteins share approximately 54% sequence homology (MmPiezo; BLASTP 2.8.1; Altschul et al. 2005) and their deletion results in embryonic lethality (Dubin et al. 2012, Li et al. 2014). Formed from three subunits, these non-selective cation channels bear little resemblance to other known channels (Coste et al. 2012, Ge et al. 2015, Wang et al. 2018). While the Piezo2 structure is yet to be determined, a recently reported cryo-electron microscopy structure for Piezo1 predicts the presence of 38 transmembrane (TM) helices in each subunit. Surrounding the central pore region, three large extended arms containing six repeated 4-TM domains are predicted to curve the lipid bilayer. This results in formation of a dome like structure that is sensitive to mechanical displacement (Guo & MacKinnon 2017, Liang & Howard 2018). Sensing changes in lateral membrane tension these channels are directly gated by membrane deformation/flattening and lateral tension induced by pressure or stretch (Coste et al. 2010, Cox et al. 2016, Guo & MacKinnon 2017, Lewis & Grandl 2015, reviewed by Wu et al. 2017a).
In the literature, Piezo2 does not appear to have a significant role in immune system function. Rather, its proposed role is to facilitate somatosensory transduction in dorsal root ganglia sensory neurons and mechano-transduction in Merkel-cells (Coste et al. 2010, Woo et al. 2014). Piezo2 expression in DP thymocytes (as provided in the ImmGen database, “abT cells group” and shown here in Figure 1.9B) is at least 10-fold lower than Piezo1 expression.

1.7.2 Piezo1 channel

Piezo1 has been proposed to play a role in stem cell lineage differentiation (Li et al. 2014, Pathak et al. 2014) perhaps having an essential role in facilitating intracellular signalling (Gottlieb & Sachs 2012). Specifically in epithelial cells, Piezo1 activation of R-Ras (a small GTPase) was implicated in regulating an inside-out signalling pathway that activates endogenous β1 integrins and hence facilitate cell adhesion (McHugh et al. 2010).

In response to mechanical strain, the channel activates rapidly (<1 ms; Gottlieb et al. 2012) and has a single channel conductance of ~30 pS (Coste et al. 2010, Gottlieb et al. 2012). It also inactivates rapidly (τ = 16.0 ± 1.2 ms; Zhao et al. 2016) via a mechanism involving the extracellular carboxy (C) terminal domain and inner pore helix (Wu et al. 2017b) and remains inactivated in the continued presence of the ongoing force stimulus (Gottlieb et al. 2012, Wu et al. 2017a). Permeable to both mono and divalent cations, the selectivity filter slightly favours Ca2+ (Zhao et al. 2016); however, the conductance of Ca2+ is lower. Ca2+ permeation appears to be retarded by hydration shell–pore interactions (15 pS for Ca2+ vs. 36 pS for Na+ at -80 mV; Gnanasambandam et al. 2015). Unlike Orai and L-type Cav
channels which have pore diameters of 6 and 6.2 Å, respectively and acid residues that coordinate shedding of the Ca\(^{2+}\) ion hydration shell as it moves through the selectivity filter (Cataldi et al. 2002, Rothberg et al. 2013), the pore diameter of Piezo 1 is predicted to be larger (~8 Å) and to allow permeation of hydrated Ca\(^{2+}\).

Primarily gated by tensile forces, Piezo1 activity may also depend upon the presence of PIP\(_2\) (Borbiro et al. 2015) and it can be inhibited by a phosphatidylserine redistribution to the outer leaflet of the PM (Tsuchiya et al. 2018). In addition, channel activity may be complexly modulated by membrane voltage (Moroni et al. 2018), by mechano-protective forces provided by cytoskeletal proteins, and by localisation to “stiffer” cholesterol-rich lipid rafts.

Figure 1.9 *Piezo1* and *Piezo2* expression in αβT lymphocyte populations

Screen shots of the relative mRNA expression of *Piezo1* (Fam38a) and *Piezo2* (Fam38b) transcripts in mice. The expression is predicted by the arbitrary numbers of transcripts given along the abscissa, where typically >120 indicates true expression (more than 95% probability), while <47 suggests the gene expression is unlikely or silent (Ericson et al. 2019). In descending order, the four DP subpopulations framed by dashed red lines are all, blasts, small resting and 69\(^+\) early positive selection. The three DP subpopulations framed by the dashed black lines are DP 69\(^-\) preselection, DP 69\(^+\) early positive selection, and DP early positive selection. Data for *Piezo1* (A; Probe set: 10582376) and *Piezo2* (B; Probe set: 10459363).
which facilitate transmission of membrane tension to increase channel gating (Cox et al. 2016, Gnanasambandam et al. 2015, Qi et al. 2015).

1.7.2.1 Piezo1 channels in thymocytes

In thymocytes, the expression of Piezo1 and whether it plays a role in thymocyte selection is yet to be determined. However, in mature T cells, Piezo1 channel activation and consequent localised Ca\(^{2+}\) flux is proposed to have an important role in promoting TCR activation and signalling (Liu et al. 2018). The Immunological Genome Project (ImmGen) database, which primarily derives its data from Affymetrix Gene microarray profiling (Heng & Painter 2008, Painter et al. 2011), indicates that, unlike Fam38b (Piezo2) the relative expression of Fam38a (Piezo1) mRNA expression is high in the abT cells group, i.e. lymphocytes that express an αβTCR (Figure 1.9). Within the DP populations (outlined by the red dashed lines) a spike in expression is noted in the DP blast population. Likewise, a spike in expression is observed in the DP CD69- subset (outlined by the black dashed lines). As CD69 expression is an early indicator of TCR activation, appearing within 2 hours of TCR stimulation (Davey et al. 1998) the DP CD69- population is likely to represent preselection thymocytes. The presence of Piezo1 protein in thymocytes is yet to be identified, and while the mRNA observation is promising (Tian et al. 2004), it does not necessarily infer of translation to Piezo1 channel expression and localisation to the PM.

Recently it has been shown that sequential application of a 10–50 pN tensile force to the TCR but not to other surface receptors such as LFA-1 results in significant localised [Ca\(^{2+}\)]\(_i\) increase (Kim et al. 2009b, Liu et al. 2014). While identification of the Ca\(^{2+}\) source was not within the scope of these papers, Liu et al. (2018) have since proposed that it is this mechanosensitive Piezo1 channel that facilitates this Ca\(^{2+}\) entry in T cell receptor signalling and activation. It is generally agreed that the TCR acts as a mechanoreceptor however the mechanism of transduction of
mechanical force to chemical signalling is not fully understood (Hu & Butte 2016, Kim et al. 2009b, Li et al. 2010).

Tangential forces in the range of 50 pN incurred during interaction of αβ chains of the TCR with the pMHC are proposed to exert torque on the CD3εγ and CD3εδ subunits necessary to trigger TCR signalling (Kim et al. 2009b). Transfer of this mechanical force to the cell membrane is suggested to involve the conformational rearrangement of the CD3ζζ juxtamembrane regions (Lee et al. 2015). Notably, the Ca²⁺ flux immediately associated with mechanically loading of the TCR is not dependent on the cytoskeletal arrangement when the applied force is cyclical (Hu & Butte 2016, Wahl et al. 2017). This finding suggests that the activation of a SAC channel, proposed to be Piezo1 in mature T-cells, is sensitive to changes in lateral membrane tension.

Of interest to this research, Doucey et al. (2003) reported that the CD3δ subunit couples the raft associated CD8 to the TCR complex and is required for positive selection of CD8⁺ lineage T cells. Specifically, this coupling is dependent upon the CD3δ interaction with the cytosolic tail of the CD8β chain (Doucey et al. 2003). Perhaps during preselection DP thymocyte–cTEC hi rosetting, this physical association provides a mechanism whereby extracellular binding of HS to the CD8 coreceptor, enhances transmembrane/intracellular CD8β mediated CD3δ conformational change, and so adjusts the transduction of concomitant pMHC-I stimulated TCR/CD3 signalling. It remains to be determined if HS (or indeed DxS) bound CD8 coreceptors induce CD3δ changes that produce torque sufficient to be detected by subunits of the CD3 receptor and facilitate a [Ca²⁺]i rise, perhaps via Piezo1 channel activation, similar to the rise reported by Kim et al. (2009b).
1.7.3 Transient receptor potential channels

Belonging to an evolutionary ancient family of channels, the mammalian TRP family comprises 28 channels which structurally contain elements reminiscent of voltage-gated K⁺ channels (reviewed by Gees et al. 2010, Kalia & Swartz 2013). While there is still much to be learnt about the physiological function of this family of channels, one proposed role for a number of these channels is to regulate immune cells (reviewed in Khalil et al. 2018, Majhi et al. 2015, Vaeth & Feske 2018).

1.7.3.1 TRP channel overview

Following the detection of an abnormal electroretinogram in a *Drosophila melanogaster* mutant (Cosens & Manning 1969), a novel Ca²⁺ channel, comprising the putative integral membrane “trp protein” (Montell & Rubin 1989), was proposed as the mechanism responsible for this transient electrical response (Hardie & Minke 1992, Hardie & Minke 1993). Since then, the mammalian TRP channel family has expanded to include 28 channels. Based on sequence homology these channels have been further grouped into 7 subfamilies. Reviewed by Nilius and Owsianik (2011), these designated subfamilies are: TRPC (the first discovered ‘canonical’; 7 members with TRPC2 being a pseudogene in humans), TRPV (vanilloid; 6 members), TRPA (ankyrin; 1 member), TRPM (melastatin; 8 members) and the more distantly related TRPP (polycystin; 3 members) and TRPML (mucolipin; 3 members). The phylogenetic relationship between TRPC, TRPV, TRPA1 and TRPM channels is shown in Appendix 4 (p.355).

These cation specific channels comprise 4 subunits and may be configured as a homomeric or a heteromeric complex assembled from subunits from one or, on occasion, different subfamilies (Bai et al. 2008, Chu et al. 2004, Fischer et al. 2014,
Li et al. 2006, Ma et al. 2010, and reviewed by Zheng 2013). Each subunit has 6 putative TM regions with the channel pore and pore loop formed between the TM5 and TM6 regions (described by Gaudet 2008 and references therein).

Mostly, the permeability ratios for divalent and monovalent cations differ between TRP channels (depicted by Gees et al. 2010; adapted in this thesis as Figure 1.10, Schrapers et al. 2018). Exceptions to this non-selective cation permeability are the Ca$^{2+}$ dependent TRPM4 and M5 channels, which conduct only monovalent cations (Launay et al. 2002, Nilius et al. 2005, Prawitt et al. 2003) and TRPV5 (originally described as ECaC) and V6 (originally CAT1), which are highly selective for Ca$^{2+}$ ($P_{\text{Ca}}:P_{\text{Na}} > 100$; Nilius et al. 2000, Yue et al. 2001). Adding complexity to the understanding of TRP channel permeability, alternative splicing sites within the pore region have been predicted to alter pore selectivity (Oberwinkler et al. 2005) and result in prolonged channel activation (reviewed by Ferreira & Faria 2016).
Figure 1.10 Ca\(^{2+}\):Na\(^{+}\) selectivity in TRP channels

Summary plot of reported Ca\(^{2+}\) to Na\(^{+}\) selectivity for different TRP channels. Here TRPV6 and TRPV5 (blue, on the left) are shown to be ~100 fold more selective for Ca\(^{2+}\) over Na\(^{+}\). In contrast, the red crosses (on the right) indicate TRPM4 and TRPM5 channels are virtually impermeable to Ca\(^{2+}\). The broad bands, shown for many of the channels, reflect the large variation in ion permeability reported in the literature (adapted from Gees et al. 2010, TRPV3 P\(_{\text{Ca}}\)/P\(_{\text{Na}}\) ~0.5, Schrapers et al. 2018).
1.7.3.2 Diverse functional domains modulate TRP channels

In addition to the diversity of ion selectivity of TRP channels, between the subfamilies moderate structural variation occurs within the intracellular amino-(N) and carboxy (C) -termini (Figure 1.11). Domains of note are the

— tandem ankyrin repeats (that vary in number from 2 to ~18), which have roles in mechano-transduction and competitive Ca²⁺–CaM/ATP binding (Erler et al. 2004, Gaudet 2008, Lee et al. 2006, Mosavi et al. 2002, Phelps et al. 2010, Sotomayor et al. 2005),

— coiled-coil motifs, which direct channel assembly and protein-protein interaction (Engelke et al. 2002, Tsuruda et al. 2006),

— proline-rich regions, which provide a binding site for Src homology 3 (SH3) domains found in proteins such as PLC-γ1 (Gaudet 2008, Yuan et al. 2003),

— IP₃R/CaM binding sites (reviewed by Eder et al. 2007, Tang et al. 2001) with a conserved “TRP box” motif, both of which reportedly regulate channel gating (Rohacs et al. 2005),

— the PSD-95/Discs-large/Zona occludens (PDZ) sequence, which forms a protein scaffold motif that regulates macromolecular complex formation (reviewed by Harteneck 2003),

— Ca²⁺ binding EF hand motifs (helix-loop-helix protein structures) that regulate channel activity in a Ca²⁺-dependent manner (Doerner et al. 2007, Kramer 2016),

— ER retention domains (Erler et al. 2004, Tsuruda et al. 2006), and

— unique enzymatic domains located in the C-terminal of TRPM6 and TRPM7 (a Mg²⁺- dependent atypical α-kinase; Ryazanova et al. 2004) and TRPM2 (an adenosine diphosphate ribose pyrophosphatase; Sumoza-Toledo & Penner 2011).
As indicated, these distinct domains facilitate subunit assembly, promote channel trafficking and cellular localisation, provide cytoskeleton anchoring sites, enhance protein-protein and lipid interaction, modulate gating via Ca\(^{2+}\) sensitisation, modulate channel phosphorylation and detect physical stimuli (Doerner et al. 2007, Garcia-Elias et al. 2015, Nilius et al. 2008, Schindl & Romanin 2007, Taberner et al. 2015, described by Venkatachalam & Montell 2007, Zheng et al. 2018). Adding to the functional diversity imparted by the cytosolic domains, variation in Trp mRNA splicing (Fischer et al. 2014, Kim et al. 2012, Lis et al. 2005, Oberwinkler et al. 2005, Zhou et al. 2013) plus the formation of heteromeric channels that exhibit distinctly different properties from their homomeric counterparts (Cheng et al. 2012, Fischer et al. 2014, Li et al. 2006) affords a broad range of characteristics to the TRP channels and imparts a vast capacity to respond to a broad range of activating mechanisms.
Figure 1.11 Variations in C- and N-termini in TRP channels

Cartoon of intracellular domains identified in the 6 TRP channel subfamilies- TRPV, TRPC, TRPA, TRPM, TRPML and TRPP. The ankyrin repeats (AnkR) in the N terminal regions of TRPA1 > TRPVs > TRPCs may enable mechanosensitivity, while coiled coil (CC) domains may enhance subunit binding. The conserved EWKFAR motif recognized as the ‘TRP box’ domain regulates gating of TRPVs, TRPCs and TRPMs. TRPC, TRPA1 and TRPP channel activity is also modulated by a Ca\(^{2+}\)-dependent CaM and IP\(_3\) receptor binding (CIRB) site or the EF hand motif. While TRPM channel insertion into the PM may be regulated by the melastatin homology domain (MHR), localisation of TRPML and TRPP is regulated by ER retention and leucine rich repeat (LRR) regions. PSD-95/Discs-large/Zona occludens (PDZ) regions and A-Kinase Anchoring Protein 5 (AKAP5) as protein binding regions facilitate macromolecular formation.
1.7.3.3 **Overview of TRP channel activation mechanisms**

A remarkable variety of physical and chemical stimuli have been reported as channel activators or inhibitors, making them important local sensors of the intracellular and extracellular environment with key roles in most physiological processes (reviews by Clapham 2003, Gees *et al.* 2010, Vrenken *et al.* 2016). Activating stimuli include 1) ligand binding, 2) thermal stimuli that range from cold through to noxious heat (reviewed in Baez *et al.* 2014), 3) non-canonical membrane voltage sensing (Palovcak *et al.* 2015), 4) intra and/or extracellular pH change (Dhaka *et al.* 2009), 5) changes in [Ca\(^{2+}\)]\(_i\) (Launay *et al.* 2002, Zurborg *et al.* 2007), 6) change in concentration of membrane-contained lipids (such as PIP\(_2\); Imai *et al.* 2012, Lucas *et al.* 2003, Nilius *et al.* 2006, Xie *et al.* 2011), polyunsaturated fatty acids (PUFAs, eg. AA; Sanaki *et al.* 2017) and epoxyeicosatrienoic acids (Watanabe *et al.* 2003b), 7) stress induced increase of reactive oxygen species (reviewed by Simon *et al.* 2013), and 8) physical stimuli induced by mechanical force or osmotic pressure fluctuation (Sato *et al.* 2013). Figure 1.12 provides a schematic summary of the polymodal gating regulation of TRPC, TRPV1, TRPA1 and TRPM channels.

In addition, posttranslational protein modification such as N-glycosylation (Dietrich *et al.* 2003, Egan *et al.* 2016, Voolstra & Huber 2014, Xu *et al.* 2006) and hydroxylation (Nagarajan *et al.* 2017), as well as phosphorylation (Yao *et al.* 2005) and the formation of macromolecular signalling complexes by some TRP channels (Adebiyi *et al.* 2011, Bandyopadhyay *et al.* 2008, Huber *et al.* 1996, Kim & Saffen 2005, Zhang *et al.* 2008) can further alter their gating properties (Hu *et al.* 2009, Palovcak *et al.* 2015, reviewed by Zheng 2013). The identification of TRP channel “networks”, which involve dozens of interacting proteins, including CaM, IP\(_3\)R, homer, FKBP, STIM1, PIP\(_3\), PKA, Src family kinases, and caveolin-1, highlights the complexity of their activation (discussed in Ambudkar & Ong...
Despite this enormous variation in activating mechanisms, it is notable that the activity of all TRP channels can be negatively regulated by \([\text{Ca}^{2+}]_i\) in a concentration dependent manner (reviewed in Gordon-Shaag et al. 2008). Not surprisingly, the mechanism of \(\text{Ca}^{2+}\) sensitisation is often complex. Notably, an increase in \([\text{Ca}^{2+}]_i\) initially increases activity of TRPA1 (Hasan et al. 2017), TRPC5 (Gross et al. 2009), TRPC6 (Shi et al. 2004), TRPV4 (Strotmann et al. 2003), TRPM2 (EC\(_{50} = 340\) nM; McHugh et al. 2003), TRPM4 and TRPM5 (Ullrich et al. 2005).

While the mechanism of sensitisation is not fully understood, \(\text{Ca}^{2+}\) dependent activation of effector molecules such as kinases, phosphatases or calmodulin have been proposed as important modulatory components in this process (reviewed in Hasan & Zhang 2018, Zhu 2005).

**Figure 1.12 TRP channel activators and inhibitors**

Schematic review of some of the reported various polymodal and non-specific stimuli that promote activation/ sensitisation or inhibition/ desensitisation of TRPC, TRPV, TRPA and TRPM channel gating. Green lines indicate activating while red indicates inhibitory.
Additional to Ca\(^{2+}\) regulation, TRP channel activity is also commonly regulated by Mg\(^{2+}\) (reviewed by Bouron et al. 2015). In common with most cells, the free [Mg\(^{2+}\)] in thymocytes is ~1 mM (Rink et al. 1982). Notably, activated TRPV3, -V6, -M1, -M6 and -M7 inward currents are reportedly suppressed by [Mg\(^{2+}\)], at concentrations of 2 mM (IC\(_{50}\)), 1 mM, 1.7 mM, 510 µM (IC\(_{50}\)) and 165 µM (IC\(_{50}\)), respectively (Chokshi et al. 2012b, Luo et al. 2012, Rampino & Nawy 2011, Voets et al. 2003, Voets et al. 2004). While the open probability of TRPV1 and TRPM3 channels is potentiated by [Mg\(^{2+}\)\(_o\)] and >2 mM [Mg\(^{2+}\)], respectively (Grimm et al. 2003, Yang et al. 2014).

Physical stimulation is another gating mechanism identified in a number of TRP channels, and interestingly, Liu and Montell (2015) propose that most TRP channels are sensitive to mechanical force. Within the literature there is considerable debate if these are mechanoreceptors. This may in part be due to the experimental challenge to distinguish between direct and indirect mechanosensitivity. As Liu and Montell (2015) argue, distinct from direct gating, forces transduced via the lipid membrane and/or the cytoskeleton (which promote channel gating) may arise downstream of a signalling cascade. Certainly in TRPA1 channels, the long ankyrin repeat region (14–18), which is proposed to act as a “spring mechanism”, may provide a force sensitive component for channel gating (Zayats et al. 2013). When transfected in HEK293 cells, exogenous TRPA1 channels were reportedly gated by a pharmacologically induced change in the composition of the lipid bilayer and the subsequent alteration in the PM curvature (Startek et al. 2018). In contrast, HEK cells expressing exogenous TRPV1 channels (which contain <10 ankyrin repeats) did not respond to a similar membrane deformation (Hill & Schaefer 2007). If not directly gated by force through membrane curvature changes, physical interaction with the elements of the cytoskeleton may provide the necessary link that imparts mechanosensitivity to TRPV1 and other proposed mechanosensitive channels like TRPV2, -V4, -C1, -

1.7.3.3.1 TRPC channel activating characteristics

It is commonly accepted that TRPC channels are gated by signalling downstream of the G-protein coupled receptors linked to Gq. This results in the activation of various isotypes of PLC to produce IP₃ and DAG. However, the mode of operation remains a debated topic, with evidence suggesting that due to store release by IP₃, they may function as either SOCE and/or receptor operated Ca²⁺ entry (ROCE) channels (Antigny et al. 2017, Jungnickel et al. 2001, Lièvremont et al. 2004, Ong et al. 2007, Plant & Schaefer 2005, Thakur et al. 2016, Trebak et al. 2003, Vannier et al. 1999, Vazquez et al. 2001, Zagranichnaya et al. 2005, Zarayskiy et al. 2007). These divergent findings are perhaps the outcome of different experimental methodologies including the cell type studied (native vs. transfected immortal cell line) or the expression level of the channel protein. With the aim of clarifying the involvement of the ER Ca²⁺ depletion in activation of TRPC channels, DeHaven et al. (2009) examined the responses in HEK-293 cells variably transfected with Stim1 and 2, Orai and Trpc cDNA and siRNA. In contrast to previous studies, they found no evidence of involvement of the ER Ca²⁺ sensor STIM1 in TRPC channel activation, thus ruling out SOCE.

Supporting ROCE, TRPC2, -C3, -C6 and -C7 channels have been found to be directly activated by the other molecule in the hydrolysis of PIP₂, namely DAG (Hofmann et al. 1999, Lemonnier et al. 2008, Lichtenegger et al. 2018, Lucas et al. 2003, Trebak et al. 2003). In addition, mechanical stress is also a proposed mechanism for activation of TRPC6, -C1 and -C3 (Anderson et al. 2013, Formigli et al. 2009, Maroto et al. 2005, Spassova et al. 2006). However, evidence for this mode of activation is not robust (Gottlieb et al. 2008). Being a pseudogene in primates, TRPC2 is less frequently studied and there is no evidence for TRPC2
mechanosensitivity. However, as with other channels, changes in membrane lipid composition and/or cytoskeleton rearrangement downstream of PLC and PI3K activation might indirectly gate TRPC2 (discussed by Liu & Montell 2015).

Less well understood are the gating mechanisms of TRPC4 and TRPC5 channels (Thakur et al. 2016). As with the other TRPC channels, stimulation of Gq proteins promotes TRPC4 and TRPC5 channel activation. However, the mechanism is not dependent on second messenger molecules downstream of PLC activation. Rather, a complex mechanism has been proposed where TRPC4 and TRPC5 channel activation involves direct gating by Gαi-protein in a PIP2 and [Ca2+]i dependent manner (Jeon et al. 2012, Thakur et al. 2016, Tsvilovskyy et al. 2009, Zholos et al. 2004).

An often reported distinctive feature of TRPC4 and 5 activation is potentiation by micromolar concentrations of lanthanides (summarised in Plant & Schaefer 2005). Considered as non-specific TRP channel inhibitors (reviewed in Bouron et al. 2015), sub-millimolar concentrations of La3+ and Gd3+ have been employed to block TRPC1, -C3, -C6, and -C7 (Halaszovich et al. 2000, Inoue et al. 2001, Okada et al. 1999). However, results from their use on native cells need to be considered with great caution as they also potently interact with NCX and SERCA (Fujimori & Jencks 1990), and may change the mechanical properties of the PM by interacting with anionic lipids (Ermakov et al. 2001), sulphates, phosphates and bicarbonates.

1.7.3.3.2 **TRPV activating channel characteristics**

TRPV channels are without doubt polymodal receptors, responding to a multitude of diverse stimuli which may be further regulated by phosphorylation (reviewed by Park et al. 2017, Yao et al. 2005). Sharing <50% homology (Hu et al. 2009), TRPV1, -V2 and to a lesser extent -V3 and -V4 (Huang et al. 2011) are
considered thermo-TRPs, opening in response to a range of threshold temperatures as described below in Table 1.2 (Caterina et al. 1999, Caterina et al. 1997, Liu et al. 2011, Peier et al. 2002, Smith et al. 2002, Watanabe et al. 2002). In contrast, TRPV5 and TRPV6, which share ~80% sequence homology, are not thermosensitive but rather, reflecting their high selectivity for Ca\(^{2+}\), are considered important channels contributing to Ca\(^{2+}\) homeostasis (Nijenhuis et al. 2005).

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<th>Table 1.2 Threshold temperatures for TRPV channel activation</th>
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While a conserved heat sensing region has been proposed in the N-terminal membrane proximal domain (MPD) of TRPV1, -V2, -V3 and -V4 (Yao et al. 2011), a single residue variation in the MPD loop region imparts use-dependent heat sensitivity to the TRPV3 channel (Liu & Qin 2017). TRPV3 has an exceptionally high value for Q\(_{10}\) (>20) and hence a very high sensitivity to temperature, particularly around physiological temperature (Xu et al. 2002). However, this temperature sensitivity appears to be complex and requires structural changes that substantially alter the gating mechanism (Liu & Qin 2017, Liu et al. 2011) and its role in thermoregulation may be minimal (Huang et al. 2011). Apart from the thermal sensing region, distinctly different thermal gating regions have been identified in TRPV1 and TRPV3. Specifically, these residues are located in the C
terminal of TRPV1 (Brauchi et al. 2007, Brauchi et al. 2006), but in the outer pore region within the helix of the sixth TM region adjoining the extracellular loop in TRPV3 (Grandl et al. 2008).

In addition to their different thermal sensitivity, TRPV1–4 channels are also differently regulated by Ca\(^{2+}\). TRPV1, -V3 and -V4 can all bind Ca\(^{2+}\)-CaM in the N terminal ankyrin repeat domain (ARD) and C terminal regions. However, the response to this Ca\(^{2+}\) dependent interaction varies between these channels. Specifically, following CaM binding, TRPV1 and -V3 channels undergo rapid desensitisation while TRPV4 activity is potentiated, at least initially (Liu et al. 2011, Numazaki et al. 2003, Phelps et al. 2010). Similarly, TRPV1, -V3 and -V4 (but not 2) are differently regulated by intracellular ATP through competitive binding at the ARD CaM binding site. When bound, ATP reduces TRPV3 sensitisation, but conversely enhances the sensitivity of TRPV1 and -V4 (Phelps et al. 2010).

Distinct from TRPV1, -V3 and -V4, TRPV2 lacks an ARD-binding site for CaM and intracellular ATP (Phelps et al. 2010). However, TRPV2 channels also undergo Ca\(^{2+}\) dependent desensitisation. A potential CaM binding domain has been identified within the C-terminus of human TRPV2 (Holakovska et al. 2011); however, its role in channel desensitisation has not been determined. Rather, the proposed mechanism leading to TRPV2 desensitisation is Ca\(^{2+}\)-dependent hydrolysis of PIP\(_2\) (Mercado et al. 2010).

The “vanilloid” designation was adopted following identification of the first member in this family (TRPV1) as a capsaicin (vanilloid) sensitive receptor (Montell et al. 2002, Szallasi & Blumberg 1996). Whilst this vanilloid sensitivity is not conserved across this subfamily, TRPV channels in general respond to a variety of pungent plant derived alkaloids, cannabinoids, inflammatory mediators (such as AA and/or its metabolites), osmolarity changes and acidic pH. Notably these stimuli are predicted to induce channel gating via different

Interestingly, the broadly used synthetic channel modulator 2-APB, which was first described as an inhibitor of IP3R (Maruyama et al. 1997), has been used to differentiate TRPV1, -V2, -V3 and -V4. In vitro results indicate that TRPV1, -V2 and -V3, but not TRPV4, are sensitive to 2-APB (Hu et al. 2004). Notably, the addition of 100 µM 2-APB potently activates TRPV1 and -V2 currents in HEK-293 cells, but in distinct contrast, the current activation in TRPV3 was minimal (Hu et al. 2006). Significantly, this poor response to 2-APB can however be uniquely potentiated if the TRPV3 channel is additionally activated by mechanisms downstream of purinergic Gq/11-protein coupled receptor stimulation (Doerner et al. 2011), by the addition of AA (Hu et al. 2006), or by priming the channel with thermal stimuli. It appears that converging and repeated stimuli, including PIP2 hydrolysis (Doerner et al. 2011) and intracellular protonation (Cao et al. 2012), uniquely sensitize TRPV3 channels. This sensitisation can reduce the temperature threshold and increase the probability of channel opening (Chung et al. 2004, Liu & Qin 2017, Liu et al. 2011).

The sensitivity of TRPV1 and -V3 to warm temperatures (~39–40°C) makes these channels ideal participants in sensing fever and stimulating the immune response. Certainly, CD8 naïve T cells cultured under the temperature of mild febrile show enhanced antigen-induced TCR activation compared to cells maintained at 37°C (Gothard et al. 2003, Mace et al. 2011, Mace et al. 2012, Smith et al. 1978). While a role for TRPV channels is yet to be established, it is conceivable that contemporaneous Ca2+ entry via thermally activated TRPV channels could synergistically potentiate TCR signalling in a manner similar to that observed downstream of LFA-1 activation (Kim et al. 2009a).
Studying a lipopolysaccharide fever model, Lai et al. (1998) found that addition of 1 µM capsaicin (a known potent TRPV1 agonist) to peripheral blood T cells caused a significant release of the inflammatory mediator substance P. While the mechanism underlying its release was not determined in this study, the authors note that other studies have reported that substance P release was evoked by an influx of Ca\(^{2+}\) triggered by capsaicin. Suggestive of activation of a capsaicin sensitive channel, the presence of functional capsaicin-sensitive channels TRPV1 and TRPV6 (Chow et al. 2007) has been detected in T lymphocytes.

1.7.3.3 TRPA1 channel activating characteristics

As indicated above, TRPA1 has a complex relationship with TRPV1. In addition to the proposed cross-talk between co-expressed TRPA1 and TRPV1 channels (Bertin et al. 2017, Schwartz et al. 2011, Staruschenko et al. 2010, Zhou et al. 2013), Fischer et al. (2014) argue that these proteins can form a functional heteromeric channel. In transfected HEK-293 cells, these authors found that the activation of TRPA1/TRPV1 heterotetramers were reminiscent of that of TRPV1, but the presence of the TRPA1 subunit suppressed channel gating.

Like TRPVs, TRPA1 can be activated by a number of capsinoids (Shintaku et al. 2012), allicin (Macpherson et al. 2005) as well as a range of highly reactive electrophilic chemicals. Commonly derived from plants, these alkaloids include nicotine, camphor, mustard oil, menthol, cinnamaldehyde, carvacrol, eugenol and thymol. Interestingly, the later five compounds are all reported agonists of TRPV3 (Bang & Hwang 2009, Wang & Wang 2017). Dependent upon the concentration, a number of these alkaloids elicit a bimodal response (Alpizar et al. 2013), which may be a consequence of off-target binding (Alvarez-Collazo et al. 2014) or reflect co-activation of the gating mechanism by Ca\(^{2+}\)-binding to the N-terminal EF hand domain (Doerner et al. 2007, Zayats et al. 2013). At low [Ca\(^{2+}\)].
the current is potentiated, but at high [Ca$^{2+}$]: channel closure ensues (Nagata et al. 2005).

Additional to this Ca$^{2+}$-dependent enhancement of ligand activation, TRPA1 is also directly activated by elevated [Ca$^{2+}$]: (EC$_{50}$ ~0.9 µM; Doerner et al. 2011, Zurborg et al. 2007), DAG (Bandell et al. 2004), AA derivatives (Motter & Ahern 2012), miRNA-711 (Han et al. 2018) and reactive oxygen species such as H$_2$O$_2$: (Andersson et al. 2008, Bessac et al. 2008). In some species, including Mus musculus (reviewed by Laursen et al. 2015), TRPA1 can be activated by temperatures below ~17°C (Chen et al. 2013, Story et al. 2003). It has also been observed with mouse TRPA1 but, in this case, this sensitivity was dependent upon point mutations within the sixth ankyrin repeat (Jabba et al. 2014).

1.7.3.3.4 TRPM activating characteristics

Of all the TRPM channels, the characteristics and regulatory mechanisms of TRPM1 are least well understood. First identified in melanocytes, this channel plays a role in cell differentiation and proliferation (Fang & Setaluri 2000). TRPM1 is also, predominantly expressed in retinal bipolar cells. In these cells, it forms a macromolecular complex with mGluR6 and GPR179 (an orphan G-protein coupled receptor, that binds HSPGs). Here its activity is suppressed by the presence of activated Goα and Gβγ proteins (Orlandi et al. 2013, Shen et al. 2012) and by [Mg$^{2+}$]. The formation of this complex and recruitment of regulator of G protein signalling (RGS) proteins, is proposed to provide a highly sensitive platform that enables discrete spatiotemporal cation influx and signal transduction (Orlandi et al. 2013). The alleviation of the voltage-independent [Mg$^{2+}$] inhibition of TRPM1, by DAG activated PKCα, is thought to further enhance the signal transmission (Rampino & Nawy 2011). Without these inhibitory mechanisms, TRPM1 is thought to be constitutively active, and while it is permeable to Ca$^{2+}$ it preferentially conducts Na$^+$ (Koike et al. 2010).
When expressed in HEK293 cells, TRPM1 functions as an ionotropic steroid receptor, a role previously identified in TRPM3, its closest relative (Harteneck 2013, Lambert et al. 2011, Wagner et al. 2008). Like TRPM1, the functional characterisation of TRPM3 is incomplete. Likely because, in both, the expression of multiple slice variants may modulate translocation to the PM, and markedly alter pore selectivity and function (Fruhwald et al. 2012, Oberwinkler et al. 2005, Xu et al. 2001). Nevertheless, the available evidence suggests that, TRPM3 is also inhibited by Gβγ-protein subunits (Quallo et al. 2017) and in common with other TRPM members, it is sensitive to mechanical and thermal stimuli.

In common with TRPA1 and -V4, TRPM4, -M5 and -M8 channels are reported to be sensitive to cool temperatures (15–25°C). While, TRPM2 and -M3 are more similar to TRPV3 and -V1, in that they are activated by innocuous (~35°C) and noxious temperatures (~40°C), respectively (reviewed in Alexander et al. 2011, Castillo et al. 2018, Diaz-Franulic et al. 2016, Vriens et al. 2011). Interestingly, similar chemical activation has also been reported for some of these channels, specifically for compounds like menthol (bimodal action on TRPA1; Karashima et al. 2007, TRPV3 agonist; Macpherson et al. 2006, TRPM8 agonist; McKemy et al. 2002), citral (bimodal action on TRPV1, -V3, -A1 and -M8; Stotz et al. 2008) and icilin (TRPA1 and -M8 agonist; Story et al. 2003).

Significantly, TRPM channels are also regulated by PIP2 which, in general, positively modulates channel activation (Badheka et al. 2015, Bousova et al. 2015, Brauchi et al. 2007, Gwanyanya et al. 2006, Holendova et al. 2012, Liu & Liman 2003, Rohacs et al. 2005, Runnels et al. 2002, Toth & Csanady 2012, Tóth et al. 2015, Xie et al. 2011). However, as also observed for other TRP channels, intracellular Ca2+-binding may affect these channels in a complex way (reviewed in Hasan & Zhang 2018, Toth & Csanady 2012) and may, in part, explain variable results, including the inhibition of TRPM7 by PIP2 (Langeslag et al. 2007). Not
unexpectedly, binding sites for PIP$_2$ and the Ca$^{2+}$-binding protein S100A1 have been identified in the N-termini of TRPM1; however, their regulatory effect on TRPM1 activity is uncertain (Jirku et al. 2015, Jirku et al. 2016).

Like most other TRP channels TRPM6 and -M7 are nonselective cation channels, but notably they are permeable to Mg$^{2+}$ and are considered important in Mg$^{2+}$ homeostasis. Unlike all other channels in this family, the ion-conducting pores of TRPM6 and -M7 are covalently coupled to an atypical serine/threonine kinase at their C-terminal. This motif is referred to as “chanzyme” as it links the ion channel with the enzymatic activity. Just how these distinct components affect channel activity is not well understood. While an independent function has been proposed (Clark et al. 2006, Krapivinsky et al. 2014, Matsushita et al. 2005, Romagnani et al. 2017), other studies have demonstrated bidirectional functional interaction. Notably, the kinase domain modulates the ion channel stability and activation, and the Mg$^{2+}$ influx regulates the enzymatic activity of the kinase (Cai et al. 2018, Cao et al. 2009, Demeuse et al. 2006, Krapivinsky et al. 2017, Thebault et al. 2008, van der Wijst et al. 2014).

Sharing 52% sequence homology, these non-redundant proteins (Jin et al. 2008a, Woudenberg-Vrenken et al. 2011) can form homomeric channels (Li et al. 2006). However, without overexpression, endogenous TRPM6 may preferentially assemble with TRPM7 to facilitate surface expression (Cai et al. 2017, Chubanov et al. 2004, Schmitz et al. 2005). Both channels are reported to be constitutively active (Monteilh-Zoller et al. 2003, Runnels et al. 2001, Suzuki et al. 2018, Voets et al. 2004) and are highly selective to divalent cations with ion permeation favouring Zn$^{2+}$ > Ba$^{2+}$ > Mg$^{2+}$ > Ca$^{2+}$ (reviewed by Bouron et al. 2015, Gees et al. 2011). Interestingly, these cations also negatively regulate channel activity, perhaps by means of electrostatic hindrance of PIP$_2$ association with the channel and/or a voltage-dependent permeation block (Chokshi et al. 2012b, Kozak & Cahalan.
Linked to their Mg\(^{2+}\) conductance, TRPM6 and -M7 channels are believed to be key regulators of cellular Mg\(^{2+}\) (Chubanov et al. 2016, Mandt et al. 2011, Ryazanova et al. 2010, Schlingmann et al. 2002, Schmitz et al. 2014, Stritt et al. 2016). In the immune system, Mg\(^{2+}\) deficiency has been linked to delayed PLC-\(\gamma1\) phosphorylation and IP\(_3\) production in human T cells (Li et al. 2011), and increased apoptosis in rat thymocytes (Gunther et al. 1984, Malpuech-Brugere et al. 1999).

Like TRPM6 and -M7, TRPM2 has been described as a chanzyme. However, this may be incorrect since Iordanov et al. (2016) recently reported that the putative “catalytic” Nudix-box domain (NUDT9-H) has no functional enzymatic activity. Rather, TRPM2 may be a ligand-gated channel. Potently activated by ADPR binding to the NUDT9-H region (Perraud et al. 2005), TRPM2 channels can be synergistically activated by reactive oxygen species such as H\(_2\)O\(_2\) and \(\beta\)-nicotinamide adenine dinucleotide (NAD\(^+\)), and also modulated by [Ca\(^{2+}\)]\(_i\) and 30 \(\mu\)M AA (Csanády & Töröcsik 2009, Hara et al. 2002, Sano et al. 2001, Zhang et al. 2018). Its sensitivity to the redox status of the cell has resulted in TRPM2 activity being commonly linked to mediation of cell death following hypoxia/anoxia (Fonfría et al. 2005, Hara et al. 2002, Perraud et al. 2005).
1.7.3.4 Pore dilation

First described for some ionotropic ATP (purinergic) receptors (P2X), sustained activation of TRPV1–V4, TRPA1 and perhaps TRPM8 appears to lead to changes in the pore configuration, which allows the diffusion of large molecular weight solutes across the PM such as N-methyl-D-glucamine (NMDG) or the fluorescent cationic dyes FM1-43 and YO-PRO-1 (Banke et al. 2010, Chen et al. 2009, Chung et al. 2008, Kittaka et al. 2017, Meyers et al. 2003, Munns et al. 2015, Zubcevic et al. 2018). Banke et al. (2010) proposed that the “pore dilation” mechanism involved conformational changes to the selectivity filter regulated by \([\text{Ca}^{2+}]_o\). Added to this, Cao et al. (2013) indicated that allosteric coupling between the selectivity filter and a lower gate, regulated by the S4-S5 and S6-TRP box linker domains provided a mechanism to expand the diameter of the lower gate. However, they did not actually detect pore dilation in the static TRPV1 configurations derived from cryo-electron microscopy.

Countering the concept of pore dilation, Li et al. (2015) argue that at least in P2X channels, these larger molecular weight solutes are always able to permeate the pore. They suggest the apparent change in selectivity was not due to pore dilation; rather, it was caused by a time-dependent change in intracellular ion concentrations and was perhaps a consequence of the electrophysiological recording technique. So, while the pore dilation phenomenon is not clearly established, it is apparent that the selectivity of some TRP channels may alter during prolonged activation.
1.7.3.5 Limitations to understanding TRP channel function

The task of investigating a role for TRP channels in native cells such as lymphocytes is complicated by 1) the variation in the biophysical properties of TRP channels described in native cells from different tissues as well as native cells from different species (Belmonte & Viana 2008, Chen et al. 2013, Grubisha et al. 2014, McIntyre et al. 2001, Saito & Tominaga 2017), 2) the likelihood that TRP channels from one or more subgroup are expressed in a cell (Dong et al. 2012, Khalil et al. 2018), and 3) the general paucity of potent and selective modulating chemicals. Disambiguation is likely a challenge when interpreting results.

1.7.3.5.1 TRP channel pharmacology is complex

Distinct from the pharmacology used to inhibit or activate voltage-gated channels, very few drugs have been identified as selective and potent modulators of TRP channel activity. To some extent, this issue stems from the fact that, following the discovery of the first TRP channel in Drosophila (Cosens & Manning 1969), sequence homology was the predominant method used to discover new TRP channels and in mammals the TRP family rapidly grew to include 28 channels by 1990. With increased understanding of the channel structure, it appears their multimodal activation characteristics (discussed in 1.7.3.3) can direct distinct activation pathways (Grandl et al. 2008, Nilius & Szallasi 2014, Zheng 2013, Zheng et al. 2018). This synergistic activation in response to distinct stimuli has made identification of drugs that target specific TRP channel activity difficult (reviewed by Gavva et al. 2008, Holzer & Izzo 2014, Moran 2018, Wu et al. 2010). Consequently, the pharmacological tools used to characterise these channels are often highly non-specific chemicals, such as 2-aminoethoxydiphenyl borate (2-APB), ruthenium red (RuR), lanthanides, flufenamic acid and plant derived cannabinoids (reviewed by Alexander et al. 2011, Belmonte & Viana 2008, Clapham 2007b, Guinamard et al. 2013).
For example, a summary of 2-APB and RuR targets is provided in Table 1.3. In most cases, the effect on the listed target channel has been described in mouse cells. In general, with regard to non-selective TRP channels, concentrations of 2-APB, varying from 10 to 500 µM have been reported to 1) inhibit TRPC2, -C3, -C5, -C6, -C7 (Lievremont et al. 2005, Viitanen et al. 2013, Xu et al. 2005), TRPM2 and -M3 (Mortadza et al. 2017, Pang et al. 2012, Togashi et al. 2008); 2) indirectly inhibit TRPM7 channel activation (Chokshi et al. 2012a); and 3) activate TRPA1 (Zhou et al. 2013), TRPM6 (Li et al. 2006) and TRPV1, -V2, and -V3 (Hu et al. 2004, Juvin et al. 2007). Its complex pharmacology also results in concentration dependent inhibitory or activating effects on Orai channel activity (Amcheslavsky et al. 2015, Prakriya & Lewis 2001) and inhibition of IP3R activation (Missiaen et al. 2001, Simkus & Stricker 2002).

Similarly as detailed in Table 1.3, RuR whilst commonly used to investigate TRPV channels has many other targets, including ryanodine receptors (RyR) located on the ER membrane (Ma 1993) and mitochondrial calcium uniporters. Specifically, in mouse pancreatic acinar cells, Wu et al. (2000) showed slow inhibition of the RyR following internal perfusion of 100 µM RuR. While in permeabilised rat mast cells, RuR has also been shown to potently inhibit mitochondrial Ca2+ uptake (Hajnóczky et al. 2006, Moore 1971). Importantly and relevant to my extracellular application, this inhibitory effect was not observed in intact rat mast cells and human hepatoma cells. The hexavalent nature of RuR is believed to slow its movement across the intact PM (Hajnóczky et al. 2006).
Table 1.3 Pharmacological effects of 2-APB and ruthenium red

Listed are channels, that are targeted by 2-APB and RuR and in most cases have been described in mouse tissue.

<table>
<thead>
<tr>
<th></th>
<th>Agonist activity</th>
<th>Antagonist activity</th>
<th>Non-channel target</th>
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<tbody>
<tr>
<td><strong>2-APB</strong></td>
<td>TRPV1, 2, 3 †</td>
<td>TRPC1, 2, 3, 4, 5, 6, 7 †</td>
<td>Directly scavenges extracellular ROS</td>
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<td></td>
<td>TRPM6 (Suzuki et al. 2017)</td>
<td>TRPM7 (Li et al. 2006), TRPM2, 3, 8 (IC₅₀=1,100,12 µM; Zholos 2010)</td>
<td>(Morihara et al. 2017)</td>
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<td></td>
<td>TRPM7 (&gt;1 mM; Li et al. 2006, Nadler et al. 2001)</td>
<td>TRPV6 (Singh et al. 2018)</td>
<td></td>
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<td></td>
<td></td>
<td>IP₃R (Maruyama et al. 1997)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>SERCA (Missiaen et al. 2001)</td>
<td>thermally activated STIM1/Orai (Liu et al. 2019)</td>
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<tr>
<td><strong>RuR</strong></td>
<td>TRPV1, 2, 3, 4, 6, A1, *</td>
<td>TRPM1 (10 µM; Shen et al. 2009)</td>
<td>Binds sialic acid residues (rat; Wieraszko 1986)</td>
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<tr>
<td></td>
<td>TRPM6 (Voets et al. 2004)</td>
<td>TRPM8 (20 µM; Zholos 2010)</td>
<td>Inhibits Ca²⁺–CaM binding (bovine; Sasaki et al. 1992); Inhibits tubulin (bovine; Deinum et al. 1985)</td>
</tr>
<tr>
<td></td>
<td>Piezo1(5.4 µM; Coste et al. 2012)</td>
<td>Catsper1, 2, 3, 4 *</td>
<td>assembly, induces disassembly (bovine; Deinum et al. 1985)</td>
</tr>
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<td></td>
<td>MCU (Moore 1971); Cav (Cibulsky &amp; Sather 1999); RyR (Wu et al. 2000);</td>
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1.7.3.5.2 Characteristics vary between native and exogenous channels

Much of the research investigating TRP channel function has been undertaken in expression systems. Typically, cultures of human embryonic kidney (HEK) 293 or Chinese hamster ovary (CHO) cells are transfected with vectors containing the DNA coding sequence for the TRP channel(s) of interest. Additional to the ectopic gene expression often forcing high protein content in typically monomeric configuration, it is notable that these cell lines also contain a number of endogenous channels, including TRP and Piezo1 (Amarouch et al. 2013, Berjukow et al. 1996, Bugaj et al. 2005, Dubin et al. 2017, He & Soderlund 2010, Vaca & Sampieri 2002).

Specifically, in immune system studies, TRP channel function has mostly been investigated using tumour-derived cell lines such as Jurkat T cells (derived from human CD4 leukemic T cells), CTLL-2 lymphocytes (cloned from C57BL/6 mouse CD8 T cells; Gillis & Smith 1977) and EL4 thymoma cells (originally derived from a carcinogen-induced C57BL/6 mouse lymphoblast tumour). Since the expression profile of ion channels may not be comparable (Kunert-Keil et al. 2006, Vandewauw et al. 2013, Wenning et al. 2011), the translation of results from immortal cell lines and expression systems to native cell function or to different tissue types, different mouse strains and/or different species may be problematic (Riccio et al. 2002). In CD4 T lymphocytes, Wenning et al. (2011) found a significant difference in the expression profile of TRP channels in Jurkat T-cells compared to native human CD4 T cells. Similarly, TRPM2 expression also differed between activated Jurkat cells and native peripheral T lymphocytes. Specifically, Kotturi and Jefferies (2005) observed mRNA expression gradually decreased in Jurkat cells compared to a two-fold increase noted for T cells.

In addition to the variation in expression, the properties of channels studied in heterologous overexpression systems may significantly differ from those in
native cells. For instance, Grubisha et al. (2014) found TRPV3 pharmacology was significantly different between recombinant rat cell lines and native channels present in a mouse keratinocyte cell line. In contrast to the activation demonstrated by exogenous TRPV3, the activation of native TRPV3 channels required the addition of markedly higher concentrations of agonists and/or exposure to disparate stimuli which act synergistically. It is likely that variation in the presence of interacting proteins and lipids between cell types alters the biophysical properties of TRP channels. Consequently, our understanding of the characteristics which influence endogenous TRP channels function in native cells is far from complete and, in thymocytes particularly, there is a scarcity of research on this topic.

While evidence of mRNA expression is not necessarily indicative of functional protein expression, it nevertheless is noteworthy that murine thymocytes contain mRNA that encodes TRPC2 and -C6, TRPM1, -M2, -M4, -M5, -M6 and -M7 and TRPV2, -V3 and -V4 (shown as Figure 1.13; adapted from Inada et al. 2006). This data provided a starting point for determining potential candidate channels that might play a role in the Dxs induced $[\text{Ca}^{2+}]$ rise observed in DP thymocytes (Weston et al. 1991). The following sections provide a more focused review of the literature; outlining characteristics specific to TRPC, TRPV, TRPM and TRPA1 channels and current evidence which suggests their functional role in T lymphocyte development, activation and death.
Figure 1.13 TRP mRNA expression during T cell development

(A) RT-PCR results showing mRNA expression of Trp genes and (house keeper) GAPDH and β-actin genes in both the thymus and isolated thymocytes from 8–12 week C57BL/6 male mice (sourced from Inada et al. 2006). (B) Summary the expression of Trp genes in thymocytes isolated by flow cytometry. Purified mRNA was amplified by RT-PCR using gene specific primer sets (+) faintly present; + present; − not detected; ND not determined (reported by Inada et al. 2006).
1.7.3.6 Proposed TRP channel roles in T lymphocytes

Without doubt, TRP channels are polymodal “receptors”. They respond to multiple disparate stimuli that may synergistically potentiate the channel activity, thereby acting as potential coincidence detectors of intra- and extracellular events and integrators of diverse activation signals (reviewed by Belmonte & Viana 2008, Hu et al. 2009, Palovcak et al. 2015, reviewed by Zheng 2013). Associated with an increased understanding of their function, there has been a rise in research papers reporting crucial roles for TRP channels in immune system development and function and, pathophysiological processes (reviews by Bertin & Raz 2016, Minard et al. 2018, Nilius et al. 2007, Vaeth & Feske 2018, Yang et al. 2009). However, to date the role of TRP channels in thymocytes is not well defined, and channels identified in immortal lymphocyte T cell lines and mature T cells may not necessarily be present or even have the same role in native thymocytes.

1.7.3.6.1 TRPCs in T lymphocytes

In human T lymphocytes, TRPC3 activation has been shown to enhance TCR dependent Ca\(^{2+}\) signalling in Jurkat T cells and naïve CD4 T cells (Pang et al. 2012, Philipp et al. 2003, Wenning et al. 2011). Specifically, Philipp et al. (2003) showed that a mutation of TRPC3 in human T cells reduced Ca\(^{2+}\) rise following TCR stimulation.

Using a Jurkat cell line, Carrillo et al. (2012) presented evidence of TRPC6 possibly forming a heteromeric TRPC3/6 channel which was activated by DAG. Notably, they observed that the TRPC3/6 opening was abolished following a methyl-ß-cyclodextrin-induced depletion of membrane cholesterol. As cholesterol is an important regulator of lipid raft integrity and cytoskeletal F-actin arrangement (Chubinskiy-Nadezhdin et al. 2013), the observed TRPC3/6 channel inhibition is
likely caused by diffusion of key modulating proteins and lipids away from the signalling domain and perhaps also actin cytoskeleton remodelling. Shown to interact with both cholesterol and TRPC6 is the integral protein podocin which may provide a regulatory “switch” enabling channel activation by either DAG or mechanical stress (Anderson et al. 2013, Huber et al. 2006). While not studied in thymocytes, it is interesting to note that in DP thymocytes, >10-fold expression of Nphs2 (podocin) has been detected (detailed in the BioGPS database, dataset: GeneAtlas MOE430, grcma, probeset: 1437605_at).

T lymphocytes harvested from Wistar rat peripheral blood also express TRPC6 > TRC3 > TRPC1. In response to induced sepsis, upregulation of TRPC6 and TRPC3 was associated with enhanced T cell apoptosis (Wu et al. 2015). In a mouse CD8 T cell line, Kim et al. (2009a) have suggested that LFA-1 dependent Ca²⁺ influx is via a DAG-activated TRPC channel. Subsequent to LFA-1/ICAM-1 interaction, this Ca²⁺ influx (shown earlier in Figure 1.3A) was found to be downstream of PLC-γ1 activation and significantly was independent of ER Ca²⁺ release. Interestingly, somewhat reminiscent of the Ca²⁺ influx under investigation in this thesis, this LFA-1/ICAM-1 associated Ca²⁺ influx has a delayed onset of approximately three minutes, which may indicate the involvement of a complex signalling mechanism and perhaps may include translocation of the channel from the endosome to the PM. It is important to note that these authors did not identify the channel involved. Reviewing the DAG sensitive TRPCs in thymocytes, it appears Trpc2 is highly expressed and to a lesser extent Trpc6 (Fig. 1.13; Inada et al. 2006). However, the presence of any DAG activated TRPC channel in mouse T cells and more specifically murine thymocytes is yet to be clarified.

Furthermore, as DAG has also been proposed to activate TRPA1 (Bandell et al. 2004) and TRPV1 (Woo et al. 2008) channels, and since its metabolite AA is an
endogenous agonist of TRPV3 and TRPV4 channels (Hu et al. 2006, Watanabe et al. 2003b), it may be argued that the influx observed down-stream of LFA-1 activation may have been caused by one of these channels and not necessarily a TRPC channel. In contrast to the lack of evidence for TRPC channels in thymocytes, functional TRPV and TRPA1 channels, however, have been detected in various T lymphocytes, including thymocytes (Bertin et al. 2014, Bertin et al. 2017, Majhi et al. 2015).

1.7.3.6.2  TRPVs in T lymphocytes

In mouse and human peripheral T cells the thermo-sensitive TRPV channels, i.e. TRPV1, -V2, -V3 and -V4 have been detected in both the PM and cytosol under resting conditions (Majhi et al. 2015). Additionally, Vassilieva et al. (2013) have reported the presence of the TRPV5 and TRPV6 proteins in peripheral lymphocytes. However, their expression level was markedly lower than that observed in tumour derived Jurkat T cells, where they are thought to play a role in tumour growth. In thymocytes, the presence of TRPV channel proteins has been less well established. While all thermo-sensing Trpv genes have been detected in murine thymocytes, with Trpv2 showing the highest expression profile (Fig. 113; Inada et al. 2006), TRPV1 and perhaps TRPV6 (Peng et al. 1999) are the only channels so far detected.

Within the TRPV family, the most extensively investigated member is TRPV1. Perhaps because of this, it is also the most commonly detected TRPV channel in T lymphocytes, including thymocytes, where it is predominantly found in cells of the CD4+ lineage. Functional TRPV1 channels identified in CD4 T cell lines from mouse, rat and human peripheral blood are generally thought to play a role in promoting cell activation and enhancing the inflammatory responses (Baker et al. 2016, Bertin et al. 2014, Saunders et al. 2007, Spinsanti et al. 2008). However, activation of TRPV1 channels detected in Wistar rat DN and SP CD4 populations
and in C57BL/6 mouse DP<sub>full</sub> thymocytes with low CD4 and CD8 expression appears to modulate the differentiation of these populations by mediating autophagy and apoptosis. In DP<sub>bright</sub> thymocytes (i.e. cells with high CD4 and CD8 expression), it is notable that TRPV1 expression was found to be negligible (Amantini et al. 2017, Amantini et al. 2004, Farfariello et al. 2012).

Additional to TRPV1, expression of TRPV2, -V3 and -V4 has been detected in human and murine primary T cells (Majhi et al. 2015, Saunders et al. 2007, Spinsanti et al. 2008). In mouse splenic T cells, TRPV4 and TRPV1 proteins reportedly co-localise with the CD4 coreceptor (Bertin et al. 2014, Chen et al. 2017a), and the activation of either of these channels was found to regulate mitogenic activity after either TCR/CD3 activation or cell stimulation with the mitogen ConA (Bertin et al. 2014, Majhi et al. 2015).

As mentioned earlier, TRPV2 activity appeared to positively regulate SOCE downstream of TCR activation (Sauer & Jegla 2006). However, this was determined in Jurkat T cells and, therefore, a similar role for TRPV2 in thymocytes may not be present. Furthermore, although the expression of Trpv2 in thymocytes appears high (Inada et al. 2006), to date, functional TRPV2 channels have only been identified in cells from thymocyte–acute lymphoblastic leukemia (T-ALL) where these channels were likely involved in volume regulation (Dobrovinskaya et al. 2015). Thus, with the exception of TRPV1, expression of functional TRPV2, -V3 and/or -V4 channels in thymocytes remains to be established.

Commonly colocalised with TRPV1, TRPA1 channels are sensitive to many of the stimuli known to also activate TRPV channels. This raises the question if TRPA1 is also present in thymocytes.
TRPA1 in T lymphocytes

The commonly reported co-expression and interaction of TRPV1 and TRPA1 channels observed in sensory neurons (Malin et al. 2011, Patil et al. 2010) has recently been confirmed in primary human CD4 T cells. In these cells, the proposed function of TRPA1 is to inhibit TRPV1 activation and thereby to suppress T cell activation (Bertin et al. 2017). To date, no evidence of TRPA1 in mouse T lymphocytes has been reported. Notably, while it does not reflect an absence of the protein, it is interesting that in rat peripheral blood lymphocytes Trpa1 expression was found to be negligible (Trevisan et al. 2014). Inada et al. (2006) in their paper examining the expression profile of Trp genes in mouse lymphocytes did not include Trpa1. However, data available in the BioGPS and ImmGen databases show slight upregulation of Trpa1 in DP thymocytes. Compared to the median determined from the RNA expression profile in a large range of mouse tissues, organs, and cell lines, TRPA1 expression is slightly increased in DP and SP CD8 thymocytes (Figure 1.14).

![Figure 1.14 Relative expression profile of murine Trpa1 in T lymphocytes](image)

Plot of the relative mRNA expression of Trpa1 during T cell maturation compared to the median determined from the RNA expression profile in a large range of mouse tissues, organs, and cell lines (BioGPS probe set 1457164_at).

The presence of TRPA1 channels in mouse thymocytes and splenic T cells has been presumed based on changes in lineage differentiation when treated with cinnamaldehyde, a TRPA1 channel agonist (Bandell et al. 2004, Fernandes et al.)
Specifically, compared to untreated thymocytes, the addition of cinnamaldehyde to murine thymocytes in single cell suspension augmented DP thymocyte differentiation to CD4 SP and CD8 SP, without apparently affecting their viability (Koh et al. 1998). This response was greatest in DP thymocytes that had higher forward scatter (indicative of larger diameter) and low side scatter measured by flow cytometry. Smaller DP thymocytes with increased side scatter (indicative of increased granularity) did not respond as strongly. Investigation of the mechanism triggered by the addition of cinnamaldehyde was beyond the scope of this research and notably, at the time of writing, the TRPA1 channel was yet to be identified (Jaquemar et al. 1999). However, with the knowledge that cinnamaldehyde activates TRPA1 channels ($IC_{50} = 9.5 \mu M$; Macpherson et al. 2006), Koh et al. (1998) results could indicate that functional TRPA1 channels are expressed during a discrete stage of thymocyte development. Equally, these findings may be due to an alternate target effect of the cinnamaldehyde such as activation of TRPV3 (0.5–5 mM; Macpherson et al. 2006) or inhibition of L-type Ca$^{2+}$ channels (Alvarez-Collazo et al. 2014).

### 1.7.3.6.4 TRPMs in lymphocytes

Detection of Trpm1, Trpm2, Trpm4, Trpm6 and Trpm7 mRNA in thymocytes has been reported (Fig. 1 13; Inada et al. 2006). Aside from Trpm7 which is moderately expressed in DP thymocytes, it is notable that extraordinarily high expression of Trpm1 in T-lymphocytes has been reported, particularly in cells that express CD8 (Figure 1.15A & B).
**Figure 1.15 Trpm7 and Trpm1 expression in T lymphocytes**

(A) mRNA expression of *Trpm7* during T cell maturation compared to the median determined from the RNA expression profile in a large range of mouse tissues, organs, and cell lines (BioGPS dataset: GeneAtlas MOE430, gcrma) probe set 1431355_s_at. (B) mRNA expression of *Trpm1*, probe set 1437455_at.

However, except for TRPM7 and perhaps TRPM2, the presence of other functional TRPM channels specifically in thymocytes remains to be established. Specifically, a loss of function in the TRPM7 ion pore significantly disturbs thymopoiesis, with a large percentage of TRPM7−/− DN thymocytes failing to differentiate to the DP stage (Jin et al. 2008a, Romagnani et al. 2017). While the mechanism causing this partial block was not determined, in activated T-cells and DT40 B lymphocytes, the loss of TRPM7 channel function reportedly impaired migration and curtailed IP₃R signalling, respectively (Kuras et al. 2012, Sahni & Scharenberg 2008). Notably, these are also critical requirements for thymocyte maturation to the DP stage (Juntilla & Koretzky 2008, reviewed in Savino et al. 2000). Furthermore, the proposed regulation of SOCE by TRPM7 channel activity would provide a crucial mechanism for regulation of Ca²⁺ homeostasis in immune cells and hence have an important role in setting an initial equilibrium within the immune system, including during thymopoiesis (Beesetty et al. 2018, Brandao et al. 2013, Faouzi et al. 2017, Mendu et al. 2018, Nadolni & Zierler 2018).
While not yet investigated in thymocytes, functional TRPM2 channels have been detected in both Jurkat and murine CD4 T cells (Beck et al. 2006, Gasser et al. 2006, Melzer et al. 2012, Sano et al. 2001). Additionally, in human primary CD4 T cells, Wenning et al. (2011) found the expression of TRPM4 increased following Ab stimulation of CD3. In the absence of stimulating antigen, T lymphocytes can be activated by an \([\text{Ca}^{2+}]_i\) rise triggered by the addition of ConA. In Jurkat cells, this ConA-induced increase in \([\text{Ca}^{2+}]_i\) has been linked to a rise in cytosolic ADPR that facilitates TRPM2 channel activation (Gasser et al. 2006, Pang et al. 2012). Of note, Buttgereit et al. (1997) observed that the addition of ConA to a rat thymocyte suspension evoked a concurrent influx of Na\(^+\) and Ca\(^{2+}\) from across the PM. Furthermore, they found that the rise in \([\text{Na}^+]_i\) and \([\text{Ca}^{2+}]_i\) was downstream of increased cellular oxidative phosphorylation and associated substrate oxidation reactions (Buttgereit et al. 1993). While they did not identify the mechanism of cation entry, their findings may arguably suggest that thymocytes also express functional TRPM2 channels.

As with TRPM2, functional TRPM4 channels are expressed in Jurkat and mature T lymphocytes; however, their presence in thymocytes has not been determined. In CD4 lineage helper type 1 and 2 cells, TRPM4 channel activation plays a significant role in differential regulation of Ca\(^{2+}\) signals. However, as TRPM4 is a non-selective for Ca\(^{2+}\), the proposed mechanism of \([\text{Ca}^{2+}]_i\) oscillations involves Na\(^+\) driving depolarization which is sufficient to reduce the driving force on Ca\(^{2+}\) entry through neighbouring Ca\(^{2+}\)-permeable channels (Launay et al. 2004, Takezawa et al. 2006, Weber et al. 2010). It is notable that in Trpm4\(^{-/-}\) mice, the thymus size and the number of mature CD4 and CD8 T cells were comparable to those from WT mice (Barbet et al. 2008). However, these broad observations do not exclude a role for TRPM4, as having a comparable number of mature T cells may not necessarily equate to a comparable TCR repertoire.
1.8 Summary

A crucial outcome of thymopoiesis is the egress of naïve T cells bearing TCRs that enrich the repertoire of the peripheral T cell pool; specifically, increasing the capacity for foreign antigen recognition while at the same time retaining tolerance of self-antigens. During thymopoiesis, testing of the diverse TCRs occurs during interactions between thymocytes and distinct populations of thymic stromal cells. These interactions facilitate activation and transduction of various signalling pathways that often depend on Ca\(^{2+}\). Perhaps as a reflection of the relatively small number of functional Ca\(^{2+}\)-permeable channels thus far identified in DP thymocytes, our understanding of Ca\(^{2+}\) entry mechanisms involved in thymopoiesis, particularly during positive selection, is incomplete. Based on the available evidence of mRNA expression, it may be that DP thymocytes variably express a multiplicity of channels, including Piezo1 and TRP channels, to orchestrate distinct [Ca\(^{2+}\)]\(_i\) rises and regulate Ca\(^{2+}\) homeostasis. While there is sparse evidence of functional expression of these channels, the mechanisms of gating, modulation and sensitisation of such channels, reported in current literature, can provide some useful information towards the identification of (a) specific channel(s).

Relevant to this thesis, a subpopulation of cTECs (i.e. cTEC\(^{hi}\)) has been found to promote rosetting of preselection DP thymocytes and induce a small [Ca\(^{2+}\)]\(_i\) rise in these thymocytes. The formation of cTEC\(^{hi}\)-thymocyte rosettes is enabled by HS engagement of minimally sialylated CD8 coreceptors at a binding site that is distinct from the MHC-I binding site. In activated DP thymocytes, the progressive sialylation of their surface receptors, including CD8, is thought to impede further rosette formation. In vitro DP–cTEC\(^{hi}\) rosette formation can also be blocked by prior application of DxS or HS to the cell suspension. It is thought
that competitive binding to the CD8 coreceptor occludes the binding site for the endogenous HS expressed.

The functional role of the $[Ca^{2+}]_i$ rise associated with preselection DP thymocyte rosetting is yet to be clarified. However, the transduction of signalling pathways activated by TCR stimulation is likely modulated by resting $[Ca^{2+}]_i$ and a concomitant $[Ca^{2+}]_i$ rise. Hence, a synergistic $[Ca^{2+}]_i$ increase when added to either a moderately strong or a negligible TCR response may alter the selection in preselection DP thymocytes that express marginally self-reactive TCR or have poor TCR-MHC avidity, respectively.

In this thesis, DxS is used to evoke a consistently observed $[Ca^{2+}]_i$ rise in DP thymocytes (Tellam & Parish 1987). This $[Ca^{2+}]_i$ rise is hypothesised to mimic the $Ca^{2+}$ influx evoked by the interaction of preselection DP thymocytes with cTEC$^{hi}$. To evoke the $[Ca^{2+}]_i$ rise, it was necessary to use large MW (500kDa) DxS which may suggest that the mechanism might involve extensive cross-linking of surface receptors and thereby impart mechanical tension. Considering the time course of the $Ca^{2+}$ influx, with its delayed onset and slow rise, channel activation is predicted to be downstream of one or several signalling cascades, rather than by direct gating by DxS.

1.8.1 Aims of study

The overall aim of this thesis is to identify the $Ca^{2+}$-permeable channel(s) activated in preselection DP thymocytes following the addition of DxS. To expand the focus in regard to function, I also aimed to identify some crucial signalling mechanisms upstream of channel activation. Recognising that DxS is not a biological ligand, it nonetheless provides an experimental paradigm that allows the investigation of signalling mechanisms and candidate channels using broad pharmacological testing in the context of flow cytometry.
To assist in the principal aim of identifying the Ca\textsuperscript{2+} permeable ion channel, there were a number of experimental objectives. These were as follows:

In Chapter 3, I will

1. determine if the channel is selectively permeable to Ca\textsuperscript{2+};

2. identify membrane receptors expressed by DP thymocytes that may be crucial to the signal transduction,

3. identify intracellular molecular components necessary for transduction of the signal cascade which leads to the channel activation, and

4. identify candidate channels based on pharmacological properties and activation characteristics.

And in Chapter 4, I will

5. investigate these “target” channels using pharmacological testing and immunocytochemistry.

1.8.2 Thesis structure

Chapter 1 of this thesis presents a review of literature considered to be pertinent to the aim of this thesis which is identifying mechanisms that induce a [Ca\textsuperscript{2+}]\textsubscript{i} rise in DP thymocytes following the addition of DxS. In Chapter 2, I provide details of the experimental protocols used in monitoring [Ca\textsuperscript{2+}], [Na\textsuperscript{+}], and [Mg\textsuperscript{2+}]; in murine DP thymocytes, the drugs used in the pharmacological testing and the method I devised for analysing data from flow cytometry. Chapter 3 presents the results of experiments which examine the 1) characteristics of the DxS induced [Ca\textsuperscript{2+}]\textsubscript{i} and [Na\textsuperscript{+}]\textsubscript{i} rises and 2) elements of the signalling cascade that appear essential/sufficient for the activation of the Ca\textsuperscript{2+} rise. Based on these results and the knowledge about Ca\textsuperscript{2+}-permeable channels described in Chapter 1, Chapter 3
includes a list of candidate channels that may be involved in the Dxs $\text{[Ca}^{2+}\text{]}$ rise. Chapter 4 presents the results of pharmacological experiments which aim to clarify which candidate channels contribute to the Dxs $\text{[Ca}^{2+}\text{]}$ rise. In Chapter 5, I summarise, discuss and contextualise the findings made in the previous chapters, identify limitations of the research and present directions for further investigation.
2 Materials and Methods

This chapter details the common materials and general methods that have been used in the experiments described in this thesis. Specific concentrations and protocols for blocking and/or activation of specific signalling pathways or candidate channels are given in the respective Materials section in Chapters 3 and 4.

2.1 Animals

Mice aged from four to ten weeks were used in this project. C57/BL/6-NCrlAnu and C57BL/6-Jackson mice were bred at the Animal Services Division of the Australian National University, while 129SvEv and 129SvEvTRPC1,3,6,7 KO mice were obtained from the Australian BioResources Pty. Ltd. with the kind permission of Professor Gary Housley, UNSW. These quadruple TRPC channel KO mice were bred from mixed C57BL/6j:129SvEv strains carrying single TRPC KO alleles and originally generated at the NIEHS, Research Triangle Park, NC, USA (TRPC1−/−; Dietrich et al. 2007, TRPC6−/−; Dietrich et al. 2005, TRPC3−/−; Hartmann et al. 2008, TRPC7−/−; Perez-Leighton et al. 2011). In these transgenic mice the respective channel has been made non-functional due to a mutation in the channel pore region. CD8β KO mice (Casey Crooks & Littman 1994) were used with the kind permission of Professor Christopher Parish, JCSMR. These mice, bred at the APF/ANU, were originally sourced from Professor Alfred Singer, NIH, USA. CD11a KO mice (strain: ENU15NIH:036:a:B6:G17) were used with the kind permission of Dr. Anselm Enders, JCSMR. The thymus from a single TRPA KO mouse, was kindly provided by Dr. E. Kheradpezhhouh, JCSMR. This animal was initially sourced from Professor Stuart Brierley, University of Adelaide, SA.
The animals were housed and transported in accordance with the guidelines laid out by the ANU Animal Experimentation Ethics committee. All mice were humanely euthanised by CO$_2$ asphyxiation in accordance with the animal welfare regulations.

### 2.2 Biochemicals

#### 2.2.1 Solutions

Commonly used chemicals for making up buffer solutions and media were sourced from various suppliers and are listed in Appendix 5 (p. 356). The recipes for commonly used solutions are detailed in Table 2.1.

HEPES buffered saline (HBS) was prepared according to the experimental requirements following the recipe described in Table 2.1. Importantly, the pH of HBS is temperature dependent, such that increasing the temperature of HBS from 7–19–37°C shifts the measured pH from 7.59 to 7.38 and 7.19, respectively. To minimise the impact of this pH shift when cells were warmed from 19 (RT) to 37°C (physiological, for flow cytometry experiments), separate aliquots of HBS were prepared on the day of the experiment. One was warmed to 37°C and pH adjusted to pH 7.3 ± 0.05, using either NaOH 1 M or HCl 1M as required. The pH of the other was similarly adjusted with the sample at RT. The osmolarity of both solutions was measured by a freezing point depression osmometer (Micro-Osmometer Autocal Type 13, Roebling) and adjusted by the addition of milliQ to 295 ± 5 mOsmol.
Table 2.1 Buffer solutions used for single thymocyte suspension

<table>
<thead>
<tr>
<th>Medium/ solution</th>
<th>Composition (mM, unless otherwise stated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dulbecco’s Phosphate Buffered Saline (PBS)</td>
<td>Ca²⁺/Mg²⁺-free. Sigma-Aldrich, St. Louis, MO</td>
</tr>
<tr>
<td>F15 MEM</td>
<td>F15 MEM powder 9.61 g/l, NaHCO₃ 2.2 g/l, in milliQ®, pH adjusted to 7.1 with HCl or NaOH prior to sterile filtering (Corning bottle cap filter 0.22 µM) which raises pH 0.1 - 0.3</td>
</tr>
<tr>
<td>HEPES buffered saline (HBS)</td>
<td>HEPES 10, NaCl 140, KCl 5, CaCl₂ 1.8, MgCl₂ 1.0, D-glucose 10, in milliQ®, titrated with NaOH to pH~ 7.3, ~295 mOsmol</td>
</tr>
<tr>
<td>HEPES buffer Na⁺ free</td>
<td>NMDG 140, KCl 5, MgCl₂ 1.0, CaCl₂ 1, D-glucose 10, HEPES 10, in milliQ®, pH ~ 7.3 adjusted with HCl, ~295 mOsmol</td>
</tr>
<tr>
<td>HEPES buffer Mg²⁺ free</td>
<td>NaCl 140, KCl 5, CaCl₂ 2.8, D-glucose 10, HEPES 10, in milliQ®, HCl pH ~ 7.3 adjusted with HCl or NaOH, ~295 mOsmol</td>
</tr>
<tr>
<td>HEPES buffer Ca²⁺ free</td>
<td>NaCl 140, KCl 5, MgCl₂ 2.8, D-glucose 10, HEPES 10, in milliQ®, HCl pH ~ 7.3 adjusted with HCl or NaOH, ~295 mOsmol</td>
</tr>
<tr>
<td>HEPES buffer with Ba²⁺</td>
<td>HEPES 10, NaCl 140, KCl 5, BaCl₂ 1.8, MgCl₂ 1.0, D-glucose 10, in milliQ®, titrated with NaOH to pH~ 7.3, ~295 mOsmol</td>
</tr>
<tr>
<td>RPMI 1640</td>
<td>Gibco® Life Technologies Carlsbad, CA</td>
</tr>
<tr>
<td>Phosphate buffered saline (PBS)</td>
<td>NaCl 150, NaPO₄ 50, in milliQ® titrated to pH 7.3</td>
</tr>
</tbody>
</table>
2.2.1.1 FACs buffer

In flow cytometry experiments, cell samples were suspended in HBS rather than the commonly used FACs buffer solution of PBS supplemented with fetal calf serum (FCS)/or BSA 5%. This was done to avert the complication of adsorption of chemicals like latrunculin B and hematoporphyrin onto albumin (Kinoshita et al. 1988, Spector et al. 1989).

2.2.2 Inhibitory and activating chemicals

In investigating the role of potential signal transduction molecules and involvement of candidate ion channels together with their selectivity, and transporters, a variety of chemicals was employed. A list of these chemicals can be found in Table 2.2 which provides the common name, International Union of Pure and Applied chemistry (IUPAC) nomenclature, catalogue number and the manufacturer.

In general, stock solutions were prepared and stored in accordance with the supplier’s recommendation. Chemicals were prepared to the maximal recommended solubility using either DMSO or milliQ, aliquoted and stored at either -80, -20 or -4°C depending on recommendation. Immediately prior to their use the stock solutions were diluted with HBS to the desired concentration. Hence, the concentration of the DMSO solvent was generally reduced to <0.01% (vol/vol). Details of their application and concentrations used will be described in greater detail in sections 3.2 and 4.2.

2.2.3 Antibodies

The primary (Ab₁) and secondary antibodies (Ab₂) and concentrations used in various flow cytometry and immunocytochemistry (ICC) experiments are listed in Table 2.3, Table 2.4 and Table 2.5. Purchased from Alomone Labs, the polyclonal anti-TRP channel Ab₁s have been validated to some extent by other
researchers. Specifically, the specificity of the anti-TRPA1 Ab has been verified in TRPA1 KO mice (Sullivan et al. 2015) while the anti-TRPC6 Ab has been validated using shTRPC6 knockdown methods in human cancer cells (Diez-Bello et al. 2019). Notably, this anti-TRPC6 (raised against aa residues 573-586 from rat TRPC6) cannot be validated in TRPC6−/− mice, where a targeted deletion of exon 7 results in expression of non-functional TRPC6 channel (specifically Trpc6tm1Lbi; Dietrich et al. 2005) but still contains the epitope (Riazanski et al. 2015). However, it has been shown to inhibit WT channel function. In electrophysiology experiments Gonzales et al. (2014) found addition of the anti-TRPC6 Ab significantly reduced the channel current.

Regarding the remaining extracellular Abs listed in Table 2.4, the specificity of the anti-TRPM6 has been verified by mock transfected HEK cells (Suzuki et al. 2017) while Yang et al. (2017) used the host IgG as a negative Ab control for anti-TRPC7 binding. Like the TRPC6 KO, the non-functional TRPC7 channel expressed in the TRPC1,3,6,7 KO mice used in my research, still contains the epitope that binds this anti-TRPC7 Ab. Hence, thymocytes from these animals do not provide a means to validate the Ab specificity. As for the anti-TRPV Abs, only the specificity of Abs for the immunogen, i.e. peptide sequence derived from the TRP protein on which the Ab was generated, has been reported (Majhi et al. 2015).

### 2.2.4 Cell-permeant cation sensitive fluorophores

For this project, the cell-permeant fluorescent indicator dyes listed in Table 2.7 were purchased in order to monitor intracellular changes in Ca²⁺, Na⁺ or Mg²⁺. The acetoxyethyl (AM) moiety conjugated to these fluorophores enables ready loading into thymocytes. Considering the excitation wavelengths of these probes (see Table 2.7), concurrent monitoring of Ca²⁺ and Na⁺ or Ca²⁺ and Mg²⁺ is possible where 4-(6-carboxy-2-indolyl)-4'-methyl-2,2'-(ethylenedioxy)-dianiline-N,N,N',N'-tetra-acetic acid tetrakis (acetoxyethyl) ester (indo-1-AM) and Asante...
NaTRIUM green 2™ acetoxymethyl (ANG2-AM) or indo-1-AM and Mag-fluo-4-AM have been loaded simultaneously.

Excited by UV light, indo-1 and the sodium binding benzofurane isophthalate acetoxymethyl (SBFI-AM) are both described as ratiometric probes. Using an excitation wavelength of 355 nm, indo-1 undergoes a distinct shift in emission fluorescence upon Ca$^{2+}$ binding ($F_{405}/F_{475}$), with the peak emission wavelength shifting from ~475 nm ($\lambda_2$) when in its unbound to ~405 nm ($\lambda_1$) when Ca$^{2+}$ is bound; however there is >30% spectral overlap. In contrast, SBFI requires a shift in the excitation wavelength to best detect the intracellular Na$^{+}$ levels, with maximal excitation at 380 in its Na$^{+}$ free state and at 340 nm in the bound state ($F_{340}/F_{380}$). Both ANG-2 and Mag-fluo-4 only increase the fluorescence intensity as they bind Na$^{+}$ or Mg$^{2+}$/Ca$^{2+}$, respectively.

With all acetoxymethyl conjugated fluorophores it is expected that intracellular esterases will cleave the AM moiety thereby converting the fluorophore to an active ionic form and at the same time trapping the dye within the cell. Nevertheless, there will be some leakage from the cell over time which can be delayed by keeping the cells at a low temperature prior to requirement. Leakage of indo-1 from thymocytes has been shown to be less than 3% over a two-hour period (Schrek et al. 1967, Tellam & Parish 1987).
### Table 2.2 Chemicals used to assess the Dxs [Ca^{2+}i] mechanism

<table>
<thead>
<tr>
<th>Common name; IUPAC name</th>
<th>(Cat. No.); Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-APB; 2-aminoethoxydiphenylborane</td>
<td>(1224); TOCRIS Bioscience, Tocris House, Bristol BS11 0QL, UK</td>
</tr>
<tr>
<td>A967079; (NE)-N-[(E)-1-(4-fluorophenyl)-2-methylpent-1-en-3-ylidene]hydroxylamine</td>
<td>(A-225); Alomone Labs, Jerusalem BioPark, Jerusalem 9104201 Israel</td>
</tr>
<tr>
<td>ATP; Adenosine 5’-triphosphate disodium salt</td>
<td>(A-2383); Sigma Aldrich St Louis, MO, USA.</td>
</tr>
<tr>
<td>Arachidonic acid; (5Z,8Z,11Z,14Z)-icosatetraenoic acid</td>
<td>(90010); Cayman chemical, Ann Arbor, MI, USA.</td>
</tr>
<tr>
<td>AMG9810; 3-(4-tert-butylphenyl)-N-(2,3-dihydro-1,4-benzodioxin-7-yl)prop-2-enamide</td>
<td>(14715); Cayman chemical</td>
</tr>
<tr>
<td>Amphotericin B; (35S)-33R-[(3-amino-3,6-dideoxy-β-D-mannopyranosyl)oxy]-1,3,5,6,9,11,17,37-octahydrxy-15,16,18-trimethyl-13-oxo-14,39-dioxabicyclo[33.3.1]nonatriacont-19,21,23,25,27,29,31-heptaene-36R-carboxylic acid</td>
<td>(11635); Cayman chemical</td>
</tr>
<tr>
<td>Cyclopiazonic acid(CPA); (2R,3S,9R)-5-acetyl-4-hydroxy-8,8-dimethyl-7,16-diaza-pentacyclo[9.6.1.0^2,9.0^3,7.0^15,18]octadeca-1(17),4,11(18),12,14-pentaen-6-one</td>
<td>(C-750); Alomone Labs</td>
</tr>
<tr>
<td>Dextran sulfate 500 kDa (Dxs) *</td>
<td>(D6001); Sigma-Aldrich. St Louis, MO, USA.</td>
</tr>
<tr>
<td>Edelfosine; 1-O-octadecyl-2-methyl-sn-glycero-3-phosphocholine</td>
<td>(3022); TOCRIS Bioscience</td>
</tr>
<tr>
<td>EGTA; Ethylene glycol-bis(2-aminoethyl ether)-N,N,N’ ,N’-tetraacetic acid</td>
<td>(E-4378); Sigma-Aldrich</td>
</tr>
<tr>
<td>FFA; flufenamic acid; 2-[[3-(Trifluoromethyl)phenyl]amino]benzoic acid</td>
<td>(F9005); Sigma-Aldrich</td>
</tr>
<tr>
<td>Gadolinium chloride</td>
<td>(93-6416); STREM chemicals, Newburyport, MA, USA</td>
</tr>
<tr>
<td>Compound</td>
<td>Formula</td>
</tr>
<tr>
<td>----------</td>
<td>---------</td>
</tr>
<tr>
<td>Gö6983; (3-[1-[3-(Dimethylamino)propyl]-5-methoxy-1H-indol-3-yl]-4-(1H-indol-3-yl)-1H-pyrrole-2,5-dione</td>
<td>(2285); Tocris Bioscience</td>
</tr>
<tr>
<td>GSK2193874; 3-[[I',4'-Bipiperidin]-1'-ylmethyl] -7-bromo-N-(1-phenylcyclopropyl)-2-[3-(trifluoromethyl)phenyl]-4-quinoline carboxamide</td>
<td>(5016); Tocris</td>
</tr>
<tr>
<td>GsMTx4; M-theraphotoxin-Gr1a</td>
<td>(STG-100); Alomone Labs</td>
</tr>
<tr>
<td>H-89; (N-[2-3-(4-bromophenyl)prop-2-enyl amino ethyl]isoquinoline-5-sulfonamide</td>
<td>(BML-E1196); ENZO life Sciences, Farmingdale, NY USA</td>
</tr>
<tr>
<td>HC-030031; 2-(1,3-dimethyl-2,6-dioxo purin-7-yl)-N-(4-propan-2-ylphenyl)acetamide</td>
<td>(11923); Cayman chemical</td>
</tr>
<tr>
<td>Hyperforin (1R,5S,6R,7S)-4-hydroxy-6-methyl-1,3,7-tris(3-methylbut-2-en-1-yl)-6-(4-methyl pent-3-en-1-yl)-5-(2-methylpropanoyl)bicyclo[3.3.1]non-3-ene-2,9-dione</td>
<td>(75650); Cayman chemical</td>
</tr>
<tr>
<td>Ionomycin (4R,6S,8S,10Z,12R,14R,16E,18R,19R,205,215)-11,19,21-Trihydroxy-4,6,8,12,14,18,20-heptamethyl-22-[25,2'R,5S,5'S]-octa hydro-5'-[(1R)-1-hydroxyethyl]-2,5'-dimethyl [2,2'- bifuran]-5-yl]-9-oxo-10,16-docosadienoic acid calcium salt</td>
<td>(Asc-370); Ascent Scientific, Islip, NY, USA</td>
</tr>
<tr>
<td>Magnesium chloride (MgCl₂)</td>
<td>(296); Ajax Chemicals Pty. Ltd. Auburn, NSW AUS</td>
</tr>
<tr>
<td>Nordihydroguaiaretic acid; 4-[4(3,4-dihydroxy phenyl)-2,3-dimethylbutyl]benzene-1,2-diol</td>
<td>(70300); Cayman chemical</td>
</tr>
<tr>
<td>NMDG; N-methyl-D-glucamine</td>
<td>(M2004); Sigma-Aldrich</td>
</tr>
<tr>
<td>Neuraminidase type II digestion enzyme *</td>
<td>Sigma-Aldrich, St Louis, MO.</td>
</tr>
<tr>
<td>Norgestimate; ((17α)-17-(Acetyloxy)-13-ethyl-18,19-dinorpregn-4-en-20-yn-3-one 3-oxime)</td>
<td>(N686000); Toronto Research Chemicals, North York, ON. CAN</td>
</tr>
<tr>
<td>NS8593; N-[1(1R)-1,2,3,4-tetrahydronaphthalen-1-yl]-1H-1,3-benzodiazol-2-amine</td>
<td>(N2538) Sigma-Aldrich</td>
</tr>
<tr>
<td>Chemical Name</td>
<td>CAS Registry Number</td>
</tr>
<tr>
<td>---------------------------------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>OAG; 1-oleoyl-2-acetyl-sn-glycerol</td>
<td>62600</td>
</tr>
<tr>
<td>PP2; 3-(4-chlorophenyl)-1-(1,1-dimethylethyl)-1H-pyrazolo[3,4-d] pyrimidin-4-amine</td>
<td>13198</td>
</tr>
<tr>
<td>Pyr3; ethyl 1-[4-(trichloroprop-2-enamido) phenyl]-5-(trifluoromethyl)-1H-pyrazole-4-carboxylate</td>
<td>3751</td>
</tr>
<tr>
<td>Ruthenium red; (RuR)[(NH$_3$)$_5$RuORu(NH$_3$)$_4$ORu(NH$_3$)$_5$]Cl$_6$</td>
<td>11103-72-3</td>
</tr>
<tr>
<td>SKF96365; 1-[(β-[3-(4-methoxyphenyl)propoxy] -4-methoxyphenethyl)-1H-imidazole hydrochloride</td>
<td>10009312</td>
</tr>
<tr>
<td>SN-6; 1-methyl-4-[[4-[(1-methylpyridin-1-ium-4-yl)amino]-phenyl]-carbamoyl]phenyl amino)quinolin-1-ium</td>
<td>2184</td>
</tr>
<tr>
<td>Tetrodotoxin (TTX); Octahydro-12-(hydroxy methyl)-2-imino-5,9:7,10a-dimethano-10aH-[1,3]dioxocino[6,5-ß]pyrimidine-4,7,10,11,12, -pentol</td>
<td>1078</td>
</tr>
<tr>
<td>Tranilast; 2-[3-(3,4-dimethoxyphenyl)-prop-2-enoylamino]benzoic acid</td>
<td>13044</td>
</tr>
<tr>
<td>YM-244769; N-[(3-aminophenyl)-methyl]-6-[4-[(3-fluorophenyl)methoxy]-phenoxy]-pyridine-3-carboxamide</td>
<td>4544</td>
</tr>
<tr>
<td>YM-58483 or BTP2; N-[4-[3,5-bis(trifluoromethyl)pyrazol-1-yl]phenyl]-4-methylthiadiazole-5-carboxamide</td>
<td>3939</td>
</tr>
</tbody>
</table>

* Kindly supplied by D. Simon Davis
Table 2.3 Primary extracellular antibodies for staining live thymocytes

Fluorochrome conjugated antibodies used to identify/gate CD4 and CD8 thymocyte populations in flow cytometry experiments. Allophycocyanin (APC), Fluorescein isothiocyanate (FITC), AlexaFluor®647 (AF647), Cyanine 7™ (Cy7)

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Isotype</th>
<th>Clone</th>
<th>Conjugate</th>
<th>Final conc (µg/mL)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4</td>
<td>Rat IgG2b,κ</td>
<td>GK1.5</td>
<td>APC</td>
<td>0.5</td>
<td>BioLegend</td>
</tr>
<tr>
<td>CD4</td>
<td>Rat IgG2a,κ</td>
<td>RM4-5</td>
<td>FITC</td>
<td>0.5</td>
<td>BD Pharmingen™</td>
</tr>
<tr>
<td>CD8a</td>
<td>Rat IgG2a,κ</td>
<td>53-6.7</td>
<td>FITC</td>
<td>1.0</td>
<td>BioLegend</td>
</tr>
<tr>
<td>CD8a</td>
<td>Rat IgGa,κ</td>
<td>53-6.7</td>
<td>AF647</td>
<td>0.4</td>
<td>BD Pharmingen™</td>
</tr>
<tr>
<td>CD8a</td>
<td>Rat IgGa,κ</td>
<td>53-6.7</td>
<td>APC-Cy7</td>
<td>0.4</td>
<td>BD Pharmingen™</td>
</tr>
</tbody>
</table>
# Table 2.4 List of primary antibodies (Abs) for immunocytochemistry

Primary extracellular anti-TRP channel antibodies used in immunocytochemistry/flow cytometry experiments.

<table>
<thead>
<tr>
<th>Anti-</th>
<th>Immunogen</th>
<th>Host, clonality</th>
<th>Lot</th>
<th>(µg/ml); dilution</th>
<th>Source (Cat #)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRPC6</td>
<td>2&lt;sup&gt;nd&lt;/sup&gt; extracellular loop aa573-586r</td>
<td>Rabbit, polyclonal</td>
<td>ACC120 AN0750</td>
<td>(0.85); 1:100</td>
<td>Alomone (ACC-120)</td>
</tr>
<tr>
<td>TRPC7</td>
<td>2&lt;sup&gt;nd&lt;/sup&gt; extracellular loop aa504-516h</td>
<td>Rabbit, polyclonal</td>
<td>ACC066 AN0102</td>
<td>(0.8); 1:100</td>
<td>Alomone (ACC-066)</td>
</tr>
<tr>
<td>TRPV2</td>
<td>1&lt;sup&gt;st&lt;/sup&gt; extracellular loop aa413-428r</td>
<td>Rabbit, polyclonal</td>
<td>ACC039 AN0202</td>
<td>(0.6); 1:75</td>
<td>Alomone (ACC-037)</td>
</tr>
<tr>
<td>TRPV3</td>
<td>1&lt;sup&gt;st&lt;/sup&gt; extracellular loop aa464-478h</td>
<td>Rabbit, polyclonal</td>
<td>ACC033 AN0225</td>
<td>(0.85); 1:100</td>
<td>Alomone (ACC-033)</td>
</tr>
<tr>
<td>TRPV4</td>
<td>3&lt;sup&gt;rd&lt;/sup&gt; extracellular loop aa647-662r</td>
<td>Rabbit, polyclonal</td>
<td>ACC124 AN0402</td>
<td>(1); 1:100</td>
<td>Alomone (ACC-124)</td>
</tr>
<tr>
<td>TRPA1</td>
<td>1&lt;sup&gt;st&lt;/sup&gt; extracellular loop aa747-760h</td>
<td>Rabbit, polyclonal</td>
<td>ACC037 AN1402</td>
<td>(8.5); 1:100</td>
<td>Alomone (ACC-037)</td>
</tr>
<tr>
<td>TRPM6</td>
<td>1&lt;sup&gt;st&lt;/sup&gt; extracellular loop aa802-815m</td>
<td>Rabbit, polyclonal</td>
<td>ACC046 AN0225</td>
<td>(8.5); 1:100</td>
<td>Alomone (ACC-046)</td>
</tr>
<tr>
<td>TRPC2 peptide</td>
<td>Isoform 4 aa 475-525</td>
<td></td>
<td>D1216</td>
<td>(4) 1:50</td>
<td>SantaCruz (sc162356P)</td>
</tr>
<tr>
<td>TRPC2</td>
<td>2&lt;sup&gt;nd&lt;/sup&gt; extracellular loop</td>
<td>Goat, polyclonal</td>
<td>H1313</td>
<td>1:50</td>
<td>SantaCruz (sc162356)</td>
</tr>
</tbody>
</table>

Alomone Labs, Har Hotzvim Hi-Teck Park, Jerusalem, Israel
Santa Cruz Biotechnology Inc., CA, USA
Table 2.5 List of secondary antibodies (Ab₂)

Secondary fluorochrome-conjugated anti-IgG antibodies used to detect primary anti-TRP channel antibodies in immunocytochemistry/flow cytometry experiments.

<table>
<thead>
<tr>
<th>Immunogen specificity</th>
<th>Host, clonality</th>
<th>Conjugate</th>
<th>Lot</th>
<th>µg/mL</th>
<th>Sourced from (Code #)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat IgG (H&amp;L)</td>
<td>Donkey polyclonal</td>
<td>FITC</td>
<td>127414</td>
<td>7.5</td>
<td>Jackson Immuno-Research (705-096-147)</td>
</tr>
<tr>
<td>Rabbit IgG (H&amp;L)</td>
<td>Donkey polyclonal</td>
<td>FITC</td>
<td>35762</td>
<td>7.5</td>
<td>Jackson Immuno-Research (711-095-152)</td>
</tr>
</tbody>
</table>

Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA

Table 2.6 Buffers and reagents used for immunocytochemistry

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blocking buffer</td>
<td>PBS + 5% donkey</td>
</tr>
<tr>
<td>Primary antibody buffer (Ab₁B)</td>
<td>PBS + 1% donkey</td>
</tr>
<tr>
<td>Secondary AbB (Ab₂ B)</td>
<td>PBS</td>
</tr>
<tr>
<td>Wash buffer</td>
<td>PBS + 0.1% donkey</td>
</tr>
<tr>
<td>Fixing agent</td>
<td>4% paraformaldehyde</td>
</tr>
</tbody>
</table>
**Table 2.7 Intracellular cation sensitive fluorescent dyes**

Details of the fluorescent indicator dyes used to monitor intracellular ions. Ex/Em λ are the reported maxima.

<table>
<thead>
<tr>
<th>Indicator (Cat. No.)</th>
<th>Binds</th>
<th>$K_d$ (mM)</th>
<th>Working range (mM)</th>
<th>Conc loaded (µM)</th>
<th>Ratiometric</th>
<th>$\lambda$ Maxima: Ex/Em (nm)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indo-1-AM (50043-1)</td>
<td>Ca$^{2+}$ free bound</td>
<td>250 (nM)$^\dagger$</td>
<td>5 yes</td>
<td>346/475</td>
<td>330/400</td>
<td>Biotium Hayward CA USA</td>
<td></td>
</tr>
<tr>
<td>Asante NaTRIUM Green-2-AM (C3512)</td>
<td>Na$^+$</td>
<td>32$^\dagger$</td>
<td>3.75–120$^\dagger$</td>
<td>5 no</td>
<td>517/540</td>
<td>TEF Labs Austin TX USA</td>
<td></td>
</tr>
<tr>
<td>SBFI-AM (sc-215841)</td>
<td>Na$^+$ free bound</td>
<td>10$^*$–20.7$^\dagger$</td>
<td>10–120$^\dagger$</td>
<td>5 yes</td>
<td>380/510</td>
<td>Santa Cruz Biotechnology CA USA</td>
<td></td>
</tr>
<tr>
<td>Mag-fluo-4-AM (M14206)</td>
<td>Mg$^{2+}$ Ca$^{2+}$</td>
<td>4.7</td>
<td>1 no</td>
<td>494/527</td>
<td>Thermo Fisher Scientific</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^\dagger$(Gryniewicz et al. 1985); $^\ddagger$(Iamshanova et al. 2016); $^*$ (Roder & Hille 2014)
2.3 Thymocyte preparation

Freshly acquired murine thymocytes were used in all experiments. The survival rate of DP thymocytes in suspension deteriorates significantly over 24 hours (Outram et al. 2002), therefore all experiments using live cells were conducted on the same day. An attempt was made to culture a comparable DP cell line from a murine thymoma cell line EL4, following the protocol described by Echevarria-Lima et al. (2005). These authors reported that a small percentage of cultured EL4 cells could be driven to express CD4 and CD8 coreceptor molecules when 75 µM indomethacin was added to the cell culture media. Notably, expression of the coreceptors was the endpoint of their experiment. For my requirement, too few cells positively stained for both coreceptors and in general the viability of the cells compared to a control EL4 cells was reduced. Furthermore, the EL4 “DP” cells did not respond to the addition of DxS.

2.3.1 Preparation of a single cell suspension

Following CO₂ asphyxiation, the thorax was opened, and the thymus removed while taking care not to rupture the heart or major blood vessels. The thymus was placed in 10 mL of ice-cold Minimum Essential Medium (MEM) in a 10 mL centrifuge tube (Technoplas). The contents were then poured into a 70 µm nylon strainer (BD Falcon™) placed in a 10 cm diameter cell culture dish. To dissociate the thymus, it was gently mashed using the plunger from a 3 mL syringe. Using a transfer pipette, the flow-through was returned to the 10 mL tube. The sample was centrifuged (300 g, 5 min, 4°C) and the supernatant discarded. The cells were washed twice, each time resuspending the pellet in 10 mL of ice-cold phosphate buffered saline (PBS) and centrifuging as before. The pellet was then resuspended in 1 mL of MEM and the cell concentration was determined.
The cell concentration was determined manually or with an automatic cell counter and viability analyser (Beckman Coulter VI-CELL™ XR, Beckman Coulter Inc, Ontario CA). To manually count, 10 µl of the cell suspension was first diluted in PBS (1:50). From this suspension, a 50 µl aliquot was further diluted with 50 µl of 0.1% trypan blue (1:2; BDH Poole Chemicals, London UK). Approximately 20 µl of the stained suspension was pipetted to fill the wells of a haemocytometer (Reichert Bright, Buffalo NY USA). The cell count was on a light microscope (BX40, Olympus Optical, Tokyo, Japan) at 40x magnification and the cell count per mL was calculated by multiplying the count/square by 10^6. For automatic cell counting, 490 µl PBS was added to a 4 mL polystyrene (PS) cup to which 10 µl of the cell suspension was then added. This sample was then run on the VI-CELL™ XR instrument.

2.3.1.1 Loading of fluorescent indicators

Stock solutions (2 mM) of the ion-sensitive indicators were prepared with dimethyl sulfoxide (DMSO). Aliquots of 2.5–5 µL were stored at -20°C protected from light. When added to the cell suspension, the final concentration was 5 µM for Ca^{2+}- and Na^{+}- or 1 µM, for Mg^{2+}-sensitive dyes. While thymocytes have been shown to be more sensitive to DMSO than splenic lymphocytes (Schrek et al. 1967), the 0.5% solvent concentration (when two fluorophores are simultaneously loaded) is not expected to have a significant cytotoxic effect within the time frame of the experiments, as they found that the mean survival of murine thymocytes incubated with 8% DMSO for 4 hours remained at 98.9 ± 4.3%.

Prior to loading, the cell concentration was adjusted to 3 x 10^7 cells/mL by the addition of MEM that was not supplemented with FBS. Once the fluorophore was added, the tube/s was/were wrapped in foil to minimise photobleaching of the fluorophore and incubated for 30–60 min at 37°C in 5% CO_2 followed by storage on ice for 20 min. Supporting the indo-1 AM loading concentration used
in this research, an examination of loading concentrations, undertaken by Tellam and Parish (1987), found incubation with 5 μM indo-1 AM for 1 h was not toxic to thymocytes suspended at 2.5 x 10^7 cells/ml. Furthermore, these authors found that it did not notably alter basal [Ca^{2+}], and it provided a “practical fluorescence signal”.

2.3.1.2 Antibody staining

When staining thymocyte surface markers, such as CD4 and CD8, the antibodies were added to the cell suspension immediately following the incubation required to load the cation-sensitive dyes. The fluorochrome conjugated antibodies and the concentrations used in staining of CD4 and CD8 are described in Table 2.3. The undiluted antibodies were added to the cell suspension which was then incubated at 4°C for 20 min. Following this incubation, residual dye and unbound antibodies were removed from suspension by washing twice with 8 mL of ice-cold PBS, centrifuged (300 g, 5 min, 4°C) and the supernatant discarded. Following the final wash, the pellet was resuspended in 1 mL HBS (pH 7.3 at 7 or 19°C), a cell count was done, and the concentration diluted to 25 x 10^6 cells/mL. The samples were immediately taken for measurement on the flow cytometer. To study the DsS-induced ionic rises, thymocytes were used on the day of preparation.

2.3.1.3 Method for preparing depleted [Ca^{2+}]_o cell suspension

Prior to pre-warming the sample, 100 μl of cells suspended in normal HBS were diluted with 400 μl of Ca^{2+} free HEPES buffer, nominally reducing [Ca^{2+}]_o to ~0.36 mM. The Ca^{2+} was then chelated by adding 1 mM EGTA to the cell suspension. Using the Maxchelator program the estimated free Ca^{2+} was calculated to be ~42 nM (http://web.stanford.edu/~cpatton/CaEGTA-NIST.htm).
2.3.2 Monitoring intracellular Ca$^{2+}$, Na$^{+}$ and Mg$^{2+}$ time course

Commonly, [Ca$^{2+}$]$_{i}$ and [Na$^{+}$]$_{i}$ were monitored simultaneously. However, in some experiments [Mg$^{2+}$]$_{i}$ instead of [Na$^{+}$]$_{i}$ was monitored. On such occasions, [Ca$^{2+}$]$_{i}$ and [Mg$^{2+}$]$_{i}$ were concurrently monitored. A BD LSRFortessa cytometer supported by BD FACSDiva™ software version 6.2 (Becton, Dickinson and Company, Franklin Lakes, NJ) was used to measure and record the time course of [Ca$^{2+}$]$_{i}$, [Na$^{+}$]$_{i}$ and [Mg$^{2+}$]$_{i}$. Table 2.8 provides the details of the lasers, detectors and filter set configuration used to excite and detect emissions from cation sensitive fluorescent indicator dyes and Ab conjugated fluorophores (used to stain for CD4 and CD8).

2.3.2.1 Ca$^{2+}$ detection using indo-1

As detailed in Table 2.8, the indo-1 fluorescence signal was detected using 379/28 nm and 505LP 530/30 nm emission filter sets for indo-1 bound and indo-1 free, respectively. Two distinct emission peaks were readily measurable. Considering the 30% spectral overlap of indo-1, this configuration results in a small “spill” of emission light into both bound (0.3%) and free (6.2%) “channels”. It also minimises the detection of uncleaved indo-1-AM fluorescence at ~480–500 nm (Lückhoff 1986).

2.3.2.2 Na$^{+}$ detection using SBFI

As detailed in Table 2.7, unlike for indo-1, SBFI is optimally excited by 2 wavelengths and detected at a single wavelength. These physical properties make SBFI a far from ideal dye to use in flow cytometry using a single laser. As well, in contrast to the considerable separation of the indo-1 emission peaks (>100 nm), there is significant spectral overlap of bound and free SBFI. Nevertheless, using the 355 nm laser to excite SBFI and the detection filter sets described in Table 2.8 differentiation of emission from free and bound dye was possible.
Manual compensation was undertaken to reduce the spectral overlap of 0.7 and 3.8% into bound and free, respectively.

Table 2.8 LSRFortessa instrument configuration

Details of the instrument set up used to excite and detect emission signals from intracellular cation sensitive fluorescent dyes and Ab conjugated fluorochromes used for detection of coreceptor expression.

<table>
<thead>
<tr>
<th>Fluorescent indicator or fluorochrome</th>
<th>Laser (nm)</th>
<th>Detector</th>
<th>Dichroic mirror Long pass (nm)</th>
<th>filter /band pass (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indo-1 free</td>
<td>UV (355)</td>
<td>A</td>
<td>None</td>
<td>379/28</td>
</tr>
<tr>
<td>Indo-1 bound</td>
<td></td>
<td>B</td>
<td>505</td>
<td>530/30</td>
</tr>
<tr>
<td>SBFI free</td>
<td>UV (355)</td>
<td>A</td>
<td>None</td>
<td>379/28</td>
</tr>
<tr>
<td>SBFI bound</td>
<td></td>
<td>B</td>
<td>None</td>
<td>450/50</td>
</tr>
<tr>
<td>ANG-2</td>
<td>Blue (488)</td>
<td>D</td>
<td>550</td>
<td>575/26</td>
</tr>
<tr>
<td>ANG-2</td>
<td>Blue (488)</td>
<td>E</td>
<td>505</td>
<td>530/30</td>
</tr>
<tr>
<td>Mag-fluo-4</td>
<td>Blue (488)</td>
<td>E</td>
<td>505</td>
<td>530/30</td>
</tr>
<tr>
<td>APC</td>
<td>Red (633)</td>
<td>C</td>
<td>None</td>
<td>670/14</td>
</tr>
<tr>
<td>FITC</td>
<td>Blue (488)</td>
<td>E</td>
<td>505</td>
<td>530/30</td>
</tr>
<tr>
<td>AF647</td>
<td>Red (640)</td>
<td>C</td>
<td>None</td>
<td>670/14</td>
</tr>
<tr>
<td>APC-Cy7</td>
<td>Red (640)</td>
<td>A</td>
<td>750</td>
<td>780/60</td>
</tr>
</tbody>
</table>

2.3.2.3 Na+ detection using ANG-2

Since in most instances, absolute values in [Na+]i were not required, a concentration change can still be monitored with a non-ratiometric dye like ANG-2 (detailed in Table 2.8). Although optimally excited by 517 nm, ANG-2 can
be sufficiently excited with a 488 nm laser. The peak amplitude of ANG-2 emission is at ~540 nm, and it increases with [Na\(^+\)]. When used alone, the ANG-2 fluorescence signal was best detected using a 505LP 530/30 nm filter set.

2.3.2.4 Simultaneous measurement of [Na\(^+\)]\(_i\) and [Ca\(^{2+}\)]\(_i\)

As both SBFI and indo-1 dyes have similar emission spectra, they cannot be used to simultaneously monitor [Ca\(^{2+}\)]\(_i\) and [Na\(^+\)]\(_i\) changes. To simultaneously monitor the two, cells were loaded with ANG-2-AM and indo-1-AM. The detection of indo-1 emission was done the same way as indicated above, but to gain better spectral separation, ANG-2 emission was measured at a longer wavelength “channel”, namely after passing a 550LP and a 575/26 nm filter set. Using this filter set decreased unwanted detection of leaked indo-1 (free) into the ANG-2 signal from ~47 to ~32%, which was further reduced by manual compensation.

2.3.2.5 Simultaneous measurement of [Ca\(^{2+}\)]\(_{ER}\) and [Mg\(^{2+}\)]\(_i\)

With the intent of monitoring [Mg\(^{2+}\)]\(_i\) changes in DP thymocytes, cells were loaded with Mag-fluo-4-AM. Mag-fluo-4 has a high sensitivity to Mg\(^{2+}\), with a \(K_d\) of ~4.7 mM. However, it is also a low affinity Ca\(^{2+}\) dye (\(K_d = 22\) µM) and since it also readily loads into the ER, it has been used to monitor [Ca\(^{2+}\)]\(_{ER}\) (Li et al. 2011).

Similar to ANG-2, Mag-fluo-4 was chosen as it can be detected simultaneously with indo-1. The excitation/emission wavelength maxima for this non-ratiometric dye is 494/527 nm. In the experiments illustrated here, the LSRFortessa 488 nm laser and 505 LP 530/30 filter set were used for excitation and emission detection.

When Mag-fluo-4 (5 µM) was loaded for 1 h at 37°C, it had readily entered the ER and bound Ca\(^{2+}\). This confounded the [Mg\(^{2+}\)]\(_i\) detection without first depleting the stores. Even with depleted stores, Mag-fluo-4 detection of [Mg\(^{2+}\)]\(_i\) was poor.
Reducing the dye loading to 30 min and its concentration to 1 µM did not alter ER uptake much. In thymocytes, this dye appears better suited for monitoring ER store Ca^{2+} rather than cytosolic Mg^{2+}.

2.3.2.6 Recording conditions

Following the setup for each dye in regard to excitation, emission filter sets and numerical compensation for spectral overlap, a template was saved in FACS Diva. New experiments were run from such saved templates. At the commencement of a new session, the voltage settings were checked to ensure optimal detection of each fluorescence signal.

To enable experiments to be conducted at 37 ± 0.5°C, a double-layer Perspex sheath was specially made to encase a 5 mL test tube (Round Bottom Falcon®) when fitted on the LSRFortessa sample injection port. Fluid from the water bath was pumped through this sheath, keeping the sample at ~37°C throughout the experiment.

Aliquots of HBS (pH adjusted for 37°C) were pipetted into a 5 mL polystyrene test and placed into a water bath set at 37°C. As required, 100 µL of the cell suspension (kept at RT, i.e. 19°C) was pipetted into a 400 µL HBS sample (or 1:5 dilution). This dilution brought the cell concentration to ~5 x 10^6 cells/mL. The sample was left to equilibrate with the bath temperature for 5 min. Following pre-warming, the tube was inserted into the Perspex sheath. During the experiment the sample was removed to add relevant chemicals and briefly vortexed before placing it back into the sheath on the cytometer. On average, the duration of the removal was ~15 s.

In this thesis, samples from single cell suspensions were acquired at a rate of ~800 events/s. Dye loading was checked by the addition either 2 µM ionomycin or 20 µM amphotericin B (AmpB) to saturate with Ca^{2+} or Na^+, respectively.
2.3.2.7 Gating hierarchy for data acquisition and analysis

BD FACSDiva software was used for acquisition setup and data acquisition. The gating hierarchy commonly used for acquisition and for data analysis using the FlowJo® V8–V10 analysis software (Tree Star Inc. Ashland, OR) are shown in Figure 2.1. Panel A (i–iv) shows the protocol used to monitor Ca\(^{2+}\) (or Na\(^{+}\), when cells were loaded with SBFI). Panel B (i–iii) shows the gating hierarchy for experiments that monitored indo-1 simultaneously with ANG2 (or indo-1 with Mag-fluo-4). In these plots the density of the observed events is represented by a colour code running from blue to red (maximum). The aim of the initial gate is to identify the thymocyte population based on the relative cell size and internal complexity of the cell, while at the same time eliminating dead cells and debris. As shown in Figure 2.1A-i & B-i, the live cell population of interest was gated (black circle) by forward scatter area (FSC-A; estimation of size) and side scatter area (SSC-A; estimation of granulation). Aggregates/doublets were excluded from this population by gating FSC-A and FSC-height (FSC-H; Figure 2.1-ii). Next, specific populations of interest, that is DP or CD8\(^{hi}\) thymocyte populations, were defined by gating on CD4 CD8 expression (DP) or CD8 expression and ANG-2 fluorescence intensity as shown in Figure 2.1A-iii & B-iii, respectively.

As illustrated in Figure 2.1A-iii, DP thymocytes form the largest population group and when using indo-1 or SBFI alone, cells were stained with anti-CD4 and anti-CD8\(^{\alpha}\) fluorochrome conjugated Abs to capture this population. In contrast, when cells were loaded with both indo-1 and ANG2, only CD8\(^{\alpha}\) staining was added. With this reduced staining it was only necessary to compensate for the fluorescence spill-over of indo-1 free (\(\lambda_2\)) into ANG2. It was decided that gating on thymocytes expressing the CD8\(^{\alpha}\) coreceptor, thus capturing small populations of pre-DP CD8 SP thymocytes and differentiated SP CD8\(^{+}\)CD4\(^{-}\) as well as the predominant DP population, would adequately identify
DxS responders. During acquisition the ratio of indo-1 and ANG2 fluorescence was monitored over time as shown in Figure 2.1A-iv & B-iv.

The FlowJo® V8–V10 kinetics platform was used to create the graphs shown in Figure 2.1A-v & B-v. These graphs plot the median fluorescence of events in each time interval which was 1–3 s depending upon duration of the acquisition.

2.3.3 Estimating the \([\text{Ca}^{2+}]_i\) and \([\text{Na}^+]_i\)

\([\text{Ca}^{2+}]_i\) and \([\text{Na}^+]_i\) were determined based on fluorescence values obtained using either a LSRFortessa™ (BD Biosciences, San Jose, CA, USA) or a Tecan microplate reader (Infinite® M200 Pro, Männedorf, Switzerland configured with Version 3.40 01/15 infinite firmware).

2.3.3.1 Determination of \([\text{Ca}^{2+}]_i\) in indo-1 loaded cells

The intracellular Ca\(^{2+}\) concentration was estimated using the following equation (Grundler et al. 2001).

\[
[\text{Ca}^{2+}]_i = K_d \frac{1 - R_{\text{min}}}{R_{\text{max}} / R - 1} \frac{S_{\lambda_2}}{S_{b2}},
\]

(Eq. 1)

where \(K_d\) is the dissociation constant of indo-1. At 37°C, this value is 250 nM (Gryniewicz et al. 1985). \(R\) is the bound/free indo-1 ratio measured during the DxS \([\text{Ca}^{2+}]_i\) plateau. \(R_{\text{max}}\) is the ratio measured (as before) under saturating Ca\(^{2+}\) conditions, induced by adding the Ca\(^{2+}\) ionophore ionomycin (3 µM) to cells suspended in normal HBS ([Ca\(^{2+}\)]_o = 1.8 mM). \(R_{\text{min}}\) was obtained by the addition of ionomycin to cells suspended in low Ca\(^{2+}\)-HBS plus 1 mM EGTA to clamp [Ca\(^{2+}\)]_o to ~40 nM. \(S_{\lambda_2}/S_{b2}\) is the measured median fluorescence intensity (from 60 s of recorded data) under saturating vs. Ca\(^{2+}\) depletion conditions in \(\lambda_2\), i.e. the emission detected after passing the 530/30 filter set.
Figure 2.1 Gating hierarchy for acquisition and analysis

A(i). The thymocyte population (black circle) is identified based on forward and side scatter area properties (FSC-A and SSC-A). A(ii). This selected population is further defined using FSC-H plotted against FSC-A to exclude aggregates. (iii) The DP thymocyte population is identified by APC and FITC (conjugated to anti-CD4 and anti-CD8 mAb, respectively) fluorescence detection. The number indicates the percentage of thymocytes contained within the gate (circle). (iv). The ratio of indo-1 (bound/free) is plotted over time for the DP thymocytes. Hiatus indicates the time when DxS was added to the sample with no detection. (v) A kinetics
plot determined from (iv) based on the median indo-1 ratio fluorescence from >600 events/s (blue). B(i-iv). Shows the hierarchy gating applied when both [Ca$^{2+}$] and [Na$^+$] are measured. (i - ii) As described in A(i-ii). (iii) Here a CD8$^{hi}$ thymocyte population is identified by AF647 (conjugated to anti-CD8 mAb) fluorescence is plotted against that of ANG-2 (black circle). (iv) Density plots based on ANG-2 fluorescence intensity and ratio of indo-1 against time. (v). [Na$^+$] and [Ca$^{2+}$] kinetics plots are derived from the events in (iv) based on the median fluorescence from >600 events/s. Consistent throughout this thesis, the control Dxs plots are colour coded blue for Ca$^{2+}$ and green for Na$^+$.

### 2.3.3.2 Determination of [Na$^+$] using ANG-2

#### 2.3.3.2.1 ANG-2 calibration

ANG-2 calibration was done using a Tecan microplate reader. Thymocytes were loaded with 1 µM ANG-2-AM following the protocol described above (2.3.1.1). To prepare samples for ANG-2 calibration, prior to the final wash the cell suspension was divided into two 10 ml Falcon tubes and following centrifugation the pellets were resuspended at a concentration of $10^6$ cells/mL, one in N-methyl-D-glucamine (NMDG) HEPES solution and the other in HBS. NMDG is used here as a replacement for Na$^+$. A range of [Na$^+$] solutions from 0–140 mM was made by serially diluting the NMDG-HEPES buffer with HBS as required. Using a Corning 96 well flat bottom polystyrene plate, 160 µl from each calibration solution was pipetted into wells and 40 µl of cells (suspended in NMDG-HEPES buffer) were added to each. This resulted in a set of [Na$^+$] values ranging from 0–112 mM. To equilibrate [Na$^+$] with [Na$^+$]o, the samples were incubated for 15 min at 37°C with 20 µM amphotericin B (AmpB).

Prior to loading the plate, the Tecan microplate reader temperature was brought to 37°C and the optimal excitation at of 515 nm and emission at 550 nm (bandwidth 9 and 20 nM, respectively) was set. The fluorescence at each [Na$^+$] was measured. These values were then normalised to the maximal value and plotted as illustrated in Figure 2.2A. $K_d$ was estimated by fitting a sigmoidal function to these data points. In the example illustrated, the $K_d$ was $37 \pm 4$ mM.
Figure 2.2 ANG-2 calibration curve

Plot of normalised fluorescence vs. [Na\textsuperscript{+}] for ANG-2 measured in thymocytes incubated with AmpB. The data points were fitted with a sigmoidal curve to yield $K_d$ (dashed lines). (B) Time courses of [Na\textsuperscript{+}]\textsubscript{i} when either Dxs or ConA was added at $t = 0$ min (green and black, respectively).

Using this value, the intracellular [Na\textsuperscript{+}]\textsubscript{i} could be estimated using equation 2 (Iamshanova et al. 2016)

$$
\left[ \text{Na}^{+} \right]_i = K_d \frac{F - F_{\text{min}}}{F_{\text{max}} - F},
$$

(Eq. 2)

where $F$ is the ANG-2 detected fluorescence intensity, $F_{\text{min}}$ is the emission signal detected when AmpB was added to thymocytes suspended in a Na\textsuperscript{+} free NMDG-HEPES buffer. $F_{\text{max}}$ was obtained by adding 20 µM AmpB to cells suspended in 140 mM Na\textsuperscript{+}-HBS. $F$ was obtained at the plateau (~15 min) after adding Dxs. These experiments were done using a BD Fortessa cytometer.

I note here that the accurate estimation the Dxs Na\textsuperscript{+} rise using the plate reader was confounded by cell death and consequent loss ANG-2 caused by incubation with AmpB. Therefore, given that the change is within the linear range of the dye
(see above), an estimation of the amplitude was made by bracketing it against the Na\(^+\) rise induced by ConA, which has been reported by Segel et al. (1979) to be \(\approx 6\) mM (Figure 2.2B).

### 2.3.4 Preparation of peripheral T cells

For peripheral T cells, following dissection of the thymus, the lymph nodes were also removed and transferred to ice-cold MEM solution. A single cell suspension was prepared following the same protocol as described for thymocytes (section 2.3.1). Two aliquots of 0.5 mL were prepared, and both were loaded with 5 \(\mu\)M SBFI-AM. Additionally, one sample had neuraminidase (from \(V.\) cholerae, final concentration 0.1 U/mL) added, while the other served as a control sample. The two tubes were wrapped in aluminium foil and incubated at 37°C in 5% CO\(_2\) as described earlier.

### 2.3.5 Immunocytochemistry

On day 1, a single cell suspension (3 \(\times\) 10\(^7\) cells/mL) was prepared following the method in section 2.3.1. Two aliquots were made and Alexa Fluor®647-CD8\(\alpha\) (1:500) was added to one to stain CD8\(^+\) thymocytes. Both samples were incubated 4°C for 20 min and then centrifuged (300 \(g\), 5 min, 4°C). The supernatant was discarded, and the pellets were resuspended in 1 mL HBS pre-warmed to 37°C. Following a cell count, the concentration was diluted to 10\(^7\) cells/mL. Aliquots of 150 \(\mu\)L from each sample were transferred to wells on a 96-round bottom plate.

The cells were fixed by the addition of 50 \(\mu\)L of 4% paraformaldehyde (final concentration 1%) and incubated for 1 h at 4°C. Next, the cells were washed twice with 200 \(\mu\)L PBS per well. The plate was centrifuged (150 \(g\), 10 min, 4°C) and the supernatant was removed. Following a second wash, the pellets were resuspended in 100 \(\mu\)l PBS/ 5% normal donkey serum to block nonspecific sites. The plate was wrapped in foil and left overnight at 4°C. Also incubated overnight.
at 4°C were the anti-TRP Ab₁–blocking peptide (BP) solutions. These solutions, prepared at a ratio of 1 µg peptide per 1 µg antibody, were placed on a rocker to facilitate Ab₁–BP binding.

All other Ab solutions were prepared on the following morning. Ab₁ and Ab₂ dilutions (detailed in Table 2.4 and 2.5) were made in PBS/ 1% donkey serum and PBS, respectively and placed on ice, while the plate was centrifuged (150 g, 10 min, 4°C). The Ab₁ solutions, Ab₁–BP or PBS/ 1% donkey serum were then added to the relevant wells and, to facilitate Ab binding, the plate was placed on a rocker for 1 h at RT. Subsequently, unbound Ab₁ was removed by washing the cells 3 times, as described above, before adding either the Ab₂ solutions or PBS to the relevant wells. The plate was placed on a rocker, leaving the cells to incubate with the Ab₂ for 1 h at RT. As before, unbound Ab₂ were removed by washing 3 times with 200 µL washing buffer and centrifuged as before. After the final wash, the pellets were resuspended in 100 µL sterile PBS, and the plate was stored at 4°C prior to analysis using flow cytometry.

2.3.5.1 Analysis of anti-TRP Ab binding using flow cytometry

The samples were run at RT on a BD LSR™II cytometer supported by FACSDiva™ software. The fluorochromes FITC and AF647 were excited using 488 and 633 nm lasers and detected using 505LP 530/30 and 660/20 filter sets, respectively. At the commencement of a new session, the voltage settings were checked to ensure optimal detection of each fluorescence signal.

As described in Gating hierarchy for data acquisition and analysis, the cell population of interest was identified based on forward and side scatter properties (Figure 2.3A-i) and to exclude debris and aggregates (A-ii). Contour plots, as shown in Figure 2.3B, were used to display the density of events relative to FITC (conjugated to the Ab₂) and AF647 (conjugated to anti-CD8α Ab) fluorescence.
intensity. The contour plots of CD8α against TRP channel expression are shown in Figure 2.3. From left to right, the four plots show the relative density and frequency of i) unstained cells, used to detect background unspecific autofluorescence, ii) anti-CD8α-AF647 plus Ab₂ (without Ab₁) stained cells, used to detect unspecific Ab₂ binding, iii) anti-CD8α-AF647 plus Ab₁–BP (anti-TRP Ab preincubated with antigen peptide) plus Ab₂ stained cells, used to check the specificity of the Ab₁ for the immunogen it was raised against, and iv) anti-CD8α-AF647 plus Ab₁ plus Ab₂ stained cells which detect binding of the primary anti-TRP channel antibody. To visually screen for the likely presence of each TRP channel, a threshold for detection of Ab₁ binding in CD8<sup>hi</sup> cells was set in plot (iii) by positioning the vertex of a quadrant gate (red) to the right of the population density. This quadrant gate was copied into plots i, ii and iv.

**Figure 2.3 Gating protocol to assess possible presence of TRP channel**
(A) Dot plots with gating hierarchy used to distinguish the cell population of interest (i) and exclude aggregates (ii). (B) Contour plots showing cell populations relative to the detection of surface expression of CD8 (conjugated AlexaFluor®647) and a TRP channel (in this case TRPV3). Abs binding was detected by Ab staining (donkey anti-rabbit IgG conjugated to FITC). The quadrant gate (red) applied in plot (iii) was copied to the other plots.

As before, the data files were analysed using the FlowJo® V8–V10 analysis software (Tree Star Inc. Ashland, OR).

2.4 Data analysis

Additional data analysis was done in Igor Pro (Versions 7 and 8; Wavemetrics, Oregon). Time series data from FlowJo kinetics plots were saved into an Excel file and imported into Igor Pro.

2.4.1 Analysis of flow cytometry time course plots

The ratio of bound over free indo-1 ($R$) was used to determine the relative change in $Ca^{2+}$. In general, this data was normalised for the ratio before the start of the experiment ($F_0$) averaged over 30 s and set to zero (Figure 2.4). The subsequent change in fluorescence ($\Delta F$) was then plotted as a time course during the experiment, with Dxs typically added at $t = 0$. FACS data for $Na^+$ and $Mg^{2+}$ fluorescence were analysed in a similar manner. The peak amplitude was equally determined as the mean over 30 s symmetrically around the peak, or typically over the last 30 s. The delay of the onset (red) of the respective rise was determined by fitting a Boltzmann equation of the following form

$$f_B = max / \left(1 + \exp^{\frac{t-h_{\text{half}}}{a}}\right)$$

to the time course, with $max$ the value of the relative peak, $h_{\text{half}}$ the time at half-maximal amplitude and $a$ the rate of rise.
The difference (in percentage) between two peak amplitudes in a set of experiments when, for example, the rise was blocked, was normalised to that of the corresponding rise with DxS (100% - control rise, blue). This is shown in Figure 2.4 (y-axis on the right) and, as an example, that when PLC was blocked (black). In this case, the difference is indicated by the red line and corresponds to ~80%.

![Figure 2.4 Time courses of normalised ΔF/F₀ caused by DxS](image)

Percentage difference (red) between DxS treated sample (blue, control) and one with an inhibitory drug added (black). Time to half maximal amplitude (t_half, black line) was determined by fitting a sigmoid to the data sets (red dash).

### 2.4.2 Statistical comparisons

Summary data is typically reported as mean ± standard error of the mean (S.E.M) with the number of experiments (n) provided. In the case of n = 3, a box plot is given with the top and bottom representing the 25th and 75th percentile. The line within the box represents the median and is typically colour coded with blue for Ca²⁺ and green for Na⁺. Where n > 3, whiskers are added to the same box indicating the range from the minimum to maximum value.
Significance level was set at 0.05. The statistical significance of the differences of means for a minimum of at least 3 separate experiments was determined by Student’s $t$-test (unpaired, two-tailed). In Figures, where asterisks used to indicate the level of significant difference, the $p$ value is <0.05 (*), <0.01 (**), <0.001 (***), and <0.0001 (****). Based on an acquisition rate of $\geq$800 events/s during flow cytometry experiments, following the removal of noise, the resultant kinetics plots generally are derived from 600 ± 23 events/s ($n = 40$ randomly opened experiments). A minimum of three repetitions was considered sufficient to determine if the samples were significantly different.

The statistical significance of the differences between two distributions of ICC samples in control and when exposed to DxS was determined using the Kolmogorov-Smirnov statistic.
3 Characterisation of the DxS \([\text{Ca}^{2+}]_i\) rise and mechanisms

3.1 Introduction

Distinct from the small transient \(\text{Ca}^{2+}\) flickers that are linked to thymocyte motility, thymocyte arrest and associated cell–cell interactions result in sustained \([\text{Ca}^{2+}]_i\) oscillations with large amplitude (Bhakta et al. 2005, Melichar et al. 2013, Ross et al. 2014). In 1987, Tellam & Parish described a sustained \([\text{Ca}^{2+}]_i\) rise that was induced in DP thymocytes (Weston et al. 1991) following the addition of DxS, but not by the addition of other smaller mol. wt. polysulfated polysaccharides. This rise was dependent upon influx of \(\text{Ca}^{2+}\) across the PM and seemingly independent of \(\text{Ca}^{2+}\) store release. Recently, Simon Davis (2015) proposed that the DxS \([\text{Ca}^{2+}]_i\) rise may mimic the \([\text{Ca}^{2+}]_i\) response observed in preselection DP thymocytes–cTEC\(^{hi}\) rosette formation. Significantly, rosette formation was markedly inhibited by the addition of anti-CD8β mAb, in CD8β deficient thymocytes, and when DxS or heparin mimetics were added to the WT thymocyte–cTEC\(^{hi}\) suspension (Simon Davis 2015). This suggests that in thymocyte–cTEC suspensions, HS binding to the CD8 coreceptor was crucial to triggering a signalling cascade that led to activation of a \(\text{Ca}^{2+}\)-permeable channel.

Without rosetting, 0.1 mg/mL heparin (a highly sulfated HS analogue) added to a thymocyte suspension was not sufficient to induce a \([\text{Ca}^{2+}]_i\) rise (Simon Davis 2015). This fact suggests that activation of other surface receptors and adhesion molecules during rosetting facilitates this \([\text{Ca}^{2+}]_i\) rise. It also suggests that in contrast to heparin, the significantly larger molecule DxS must interact with other surface molecules in addition to CD8β. Interestingly, while \(\alpha\beta\text{TCR–MHC-I}\) interactions have been shown to enhance the \([\text{Ca}^{2+}]_i\) rise associated with rosetting, Simon Davis (2015) observed TCR/CD3 signalling via ZAP-70 activation was not
necessary in facilitating the DxS \([\text{Ca}^{2+}]\) rise. In thymocytes derived from ZAP-70 deficient mutant mice, he found that the DxS \([\text{Ca}^{2+}]\) rise was unchanged. While this finding rules out the involvement of the ZAP-70 signalling pathway, it does not necessarily exclude any CD3 involvement with DxS. Upstream of ZAP-70 phosphorylation of the CD3ζ chains, avid physical association between the CD8 coreceptor β chain and CD3δ may facilitate a conformational change in CD3 in response to CD8β stimulation (Doucey et al. 2003). Associated with this conformational change is the proposed SLP-76 recruitment of the adaptor protein Nck to the CDε chain (Barda-Saad et al. 2004, de la Cruz et al. 2011). In turn, Nck engages phosphorylated SLP-76, ADAP and WASP to the inside-out signalling cascade required for LFA-1 activation and to the LAT-nucleated macromolecular signalling complex, a necessary step in the orchestration of PLC-γ1 activity (Gil et al. 2002).

Notably, in thymocytes derived from SLP-76 deficient mice the DxS \([\text{Ca}^{2+}]\) rise was found to be significantly diminished (Simon Davis 2015) suggesting a crucial role for this adaptor protein. While SLP-76 is best phosphorylated by ZAP-70, it may also be phosphorylated by Lck (Y423/426; Wardenburg et al. 1996). This may be sufficient to allow suboptimal TCR stimulation to evoke an LFA-1 dependent \([\text{Ca}^{2+}]\) rise like the one described by Kim et al. (2009a; Figure 1.3A; black trace).

If the DxS \([\text{Ca}^{2+}]\) rise mimicked a physiological \([\text{Ca}^{2+}]\) rise which functions to modify or fine tune the selection processes by “priming” of some preselection thymocytes, then identifying the mechanisms which are activated upstream of the DxS-induced \([\text{Ca}^{2+}]\) influx will add to our understanding of T cell development by revealing critical features of selection. Furthermore, exploring the characteristics and mechanisms which modulate the DxS-induced \([\text{Ca}^{2+}]\) influx observed in DP thymocytes may help to identify the ion channel(s) involved.
In this chapter, I am presenting results which further characterize the DxS $[\text{Ca}^{2+}]_i$ rise described. I will show that the addition of DxS results not only in a $\text{Ca}^{2+}$ but also in a concomitant $[\text{Na}^+]_i$ rise and that activation of the transmembrane flux is indeed independent of $\text{Ca}^{2+}$ release from ER stores. The DxS mediated signalling mechanism is proposed to mimic the HS signalling pathways described in 1.3.1.6.1 (p. 52) and depicted in Figure 1.4. Therefore, in this chapter I also present the results from flow cytometry experiments which aimed to identify crucial receptors, kinases and other proteins that facilitate the transduction of the relevant signalling pathways leading to subsequent activation of an unspecific cation permeable channel or channels sustaining the two rises. Results from this chapter will be used in narrowing down a list of putative channel(s) presented in the following chapter.

3.2 Materials

Details of the solutions and chemicals commonly used in cell preparation and flow cytometry experiments can be found in Chapter 2. Table 3.1 provides details of the concentrations of the various chemicals used and when they were added to the sample. Unless otherwise stated, DxS was added to the cell suspension after at least one minute of recording. In control experiments where the effect of the chemical without the addition of DxS was tested, the chemicals were also added to the cell suspension at $t = 0$ min and at the concentrations stated in Table 3.1.
Table 3.1 List of chemicals

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Putative target</th>
<th>Mechanism</th>
<th>Protocol</th>
<th>Conc. (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caffeine</td>
<td>Phosphor-diesterase</td>
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<td>5 min pre-incub.</td>
<td>400</td>
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<td>ConA</td>
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<td>mitogenic activator</td>
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<td>SERCA</td>
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</tr>
<tr>
<td>EGTA</td>
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<td>chelator</td>
<td>Added at $t = -1$ min</td>
<td>5 mM or 100</td>
</tr>
<tr>
<td>Edelfosine</td>
<td>PLC</td>
<td>inhibitor</td>
<td>Added at $t = -1$ min</td>
<td>1</td>
</tr>
<tr>
<td>FFA</td>
<td>TRPC3</td>
<td>inhibitor</td>
<td>5 min pre-incub. or at 12 min</td>
<td>100</td>
</tr>
<tr>
<td>Gö6983</td>
<td>PKC</td>
<td>inhibitor</td>
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</tr>
<tr>
<td>GsMtx4</td>
<td>SAC channels</td>
<td>inhibitor</td>
<td>Added at $t = -5$ min</td>
<td>3.5</td>
</tr>
<tr>
<td>H-89</td>
<td>PKA</td>
<td>inhibitor</td>
<td>Added at $t = -15$ min</td>
<td>0.5</td>
</tr>
<tr>
<td>Ionomycin</td>
<td>Raises $[\text{Ca}^{2+}]_i$</td>
<td>ionophore</td>
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<td>PI3K</td>
<td>inhibitor</td>
<td>30 min pre-incub. at 37°C</td>
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</tr>
</tbody>
</table>
3.3 Characteristics of the Dxs-induced cation influx

3.3.1 Reproduction of Dxs $[\text{Ca}^{2+}]_i$ rise in DP thymocytes

In investigating the characteristics of the $[\text{Ca}^{2+}]_i$ rise, the first step was to determine if the properties of the $[\text{Ca}^{2+}]_i$ rise detected by flow cytometry were comparable to that described by Tellam and Parish (1987) which was based on measurements by a spectrometer.

After pre-warming the sample cells to 37°C for 5 min in a water bath, the addition of 100 µg/mL Dxs to a thymocyte suspension resulted in a robust rise in $[\text{Ca}^{2+}]_i$, which started after ~5 min, slowly reached a peak after 15 min (Figure 3.1A, blue), and maintained a plateau for at least another 15 min (not shown). Here, the relative change in $[\text{Ca}^{2+}]_i$ is plotted over time after the addition of Dxs at $t = 0$ min (grey arrowhead) and after normalising for the baseline to be zero.
The black trace shows a typical time course for a control sample when no Dxs was added to the suspension. The ochre trace was obtained when 5 mM EGTA was added to the suspension (normal HBS) before the addition of Dxs (t = -1 min), indicating that the rise is dependent upon a transmembrane Ca$^{2+}$ influx into the thymocytes and that the background Ca$^{2+}$ in these cells is dependent on a Ca$^{2+}$ leak either across the PM or from stores. The peak amplitude of this [Ca$^{2+}$], rise was well within the linear range of the indo-1 dye as determined by the addition of 2 µM ionomycin during the plateau period (Figure 3.1D, black arrow), which still resulted in a ~5-fold increase of the normalised ratio.

The [Ca$^{2+}$] concentration of the Dxs induced rise, as detected by flow cytometry, was estimated using Eq. 1 (see Methods 2.3.3.1) and compared with the result reported by Tellam and Parish (1987). Based on a resting [Ca$^{2+}$] of ~110 nM (Hesketh et al. 1983, Ross & Cahalan 1995, Tellam & Parish 1987), the Dxs [Ca$^{2+}$] rise was found to be 165 ± 11 nM (n = 5). This value is not different to that reported by Tellam & Parish (1987; 146 ± 23 nM, n = 11, p = 0.28). For comparison, their figure is redrawn here (Figure 3.1B), where in blue, the Ca$^{2+}$ rise is shown as measured on the Perkin Elmer LS-5 luminescence spectrometer and in ochre, after 5 mM EGTA had been added to the suspension. On the left, R indo-1 is plotted and on the right, the respective estimated Ca$^{2+}$ concentration.

Notably, in the original time course, there was a small initial Ca$^{2+}$ rise within about 2 min, which was also abolished when the [Ca$^{2+}$]o was chelated with EGTA (see Fig.3.1B, ochre; Tellam & Parish 1987). This small early rise was not apparent in the traces obtained with flow cytometry.

In agreement with the later findings reported by Weston et al. (1991), the [Ca$^{2+}$] rise was indeed restricted to thymocytes in the DP population as shown in Figure 3.1C (blue). A smaller, highly variable rise was observed in CD8 SP thymocytes (grey). This finding was inconsistent and most likely an artefact of population
gating (Figure 3.1C, inset), which may have included early transitional CD4<sup>int</sup> CD8<sup>hi</sup> thymocytes in this population. An even smaller rise was seen in CD4 SP cells (pink), but the [Ca<sup>2+</sup>]<sup>i</sup> in the DN subset (red) was no different to the control when no DxS was added (black). With the exception of the early rise not present in cytometry data, the findings so far are consistent with the idea that there was no difference between the rise measured with the cytometer vs. that with the spectrometer and suggests that with my methods, the same Ca<sup>2+</sup> rise was tracked reliably.

### 3.3.2 The DxS [Ca<sup>2+</sup>] rise measured early vs. late was comparable

As most cytometry experiments were conducted over a period of three to four hours, it was important to determine if the DxS [Ca<sup>2+</sup>] rise could still be reliably activated in samples that were “freshly” prepared vs. ones that had been stored on ice for more than two hours. It was noticed that keeping the cells on ice for prolonged periods caused a gradual increase in the resting [Ca<sup>2+</sup>]<sup>i</sup> evidenced by the fact that during a subsequent experiment there was an increased R of indo-1 fluorescence. In these samples, the amplitude of the DxS [Ca<sup>2+</sup>] rise was also noticeably smaller. However, keeping the cells at RT (~19°C in the cytometry facility) alleviated this problem.

Over a period of four hours, and when the cell suspension was stored at RT, no significant difference was found between the indo-1 R obtained from early and late samples (Figure 3.1E; n = 26; p = 0.89). In this figure, one of two samples was measured early (left) and the other late (right). The respective values for the same preparation are joined by a line with the respective box-and-whisker plots overlaid.
Figure 3.1 Characterisation of the Dxs \([\text{Ca}^{2+}]_i\) rise

(A) Time courses of Dxs \([\text{Ca}^{2+}]_i\) under normal conditions (blue) when Dxs was added at \(t = 0\) (grey arrowhead). Controls without Dxs (black) and with Dxs in the presence of 5 mM EGTA extracellularly (ochre). (B) Time courses obtained in a spectrometer by Tellam and Parish (1987) for Dxs (blue) and in the presence of 5 mM EGTA (ochre). (C) \([\text{Ca}^{2+}]_i\) time courses evoked by Dxs added at \(t = 0\) (grey arrow) for the 4 different thymocyte populations based on low vs. high gating on CD4 and CD8 (inset) with DP cells (blue), CD8 SP (grey), CD4 SP (pink) and DN (red). Control without Dxs in black. (D) Time course of Dxs \([\text{Ca}^{2+}]_i\) under normal conditions \((t = 0\) min) with subsequent addition of 2 \(\mu\)M ionomycin at \(t = 15\) min (black arrow). (E) Plots of peak amplitudes \((n = 26)\) of \([\text{Ca}^{2+}]_i\) from the same preparation obtained early (left) and late (right) with data pairs joined by a line and box-and-whisker plot superimposed.

Importantly results presented in 3.3.1–3.3.3, show that the Dxs induced \([\text{Ca}^{2+}]_i\) rise is repeatable and consistent over experimental sessions lasting up to 4 h. In the following sections I demonstrate how the ‘Dxs related’ results may be altered by subtle changes in the background \([\text{Ca}^{2+}]_i\) caused by drugs or changes in temperature. These stimulatory mechanisms may include thermally activated STIM1 (Xiao et al. 2011) or drugs such as the mitogen Concanavalin A, or channel inhibitors such as Pyr3, and flufenamic acid which evoke a sustained rise in
[Ca\textsuperscript{2+}]. Depending upon when these stimuli are applied, they either abolish or potentiate the DxS induced [Ca\textsuperscript{2+}]_i rise.

### 3.3.3 The mechanism is sensitive to elevated background [Ca\textsuperscript{2+}]_i:

In addition to being very sensitive to temperature, the mechanism is also dependent on background [Ca\textsuperscript{2+}]_i. Significantly, while investigating the relationship between the rises induced by ConA and DxS, I noticed that a stable elevation of the background [Ca\textsuperscript{2+}]_i by ~60 nM prior to the addition of DxS occluded any subsequent DxS Ca\textsuperscript{2+} rise. As also found by Tellam and Parish (1987), the addition of 10 µg/ml ConA during the plateau phase of the DxS [Ca\textsuperscript{2+}]_i rise still evoked a transient additive [Ca\textsuperscript{2+}]_i rise (Figure 3.2A; red arrowhead on blue). However, this time course was not only faster than when added on its own (Figure 3.2A; red arrow at *t* = 0 min, red), but also much smaller. This suggests that ConA causes a rise via a different mechanism, but that the two are not necessarily independent of each other. In addition, I have also been able to show that in contrast to the DxS rise, ConA can still evoke a much smaller Ca\textsuperscript{2+} transient under conditions when [Ca\textsuperscript{2+}]_o is nominally 0 mM with 100 µM EGTA (red arrowhead on ochre). This suggests that ConA involves Ca\textsuperscript{2+} release from stores.

The unexpected finding, however, was that, under conditions when cells were pre-incubated with ConA for 15 min, the DxS [Ca\textsuperscript{2+}]_i rise (blue, without ConA as control) was abolished (Figure 3.2B; brown). Note that in contrast to most other figures, I am presenting this data without normalisation of R (raw). This is to clarify that background [Ca\textsuperscript{2+}]_i was elevated before the addition of DxS by about 60 nM. This experiment was repeated 5 times and the block was highly significant (*p* < 0.0001). This finding suggests that prior elevation of [Ca\textsuperscript{2+}]_i can occlude the transmembrane DxS rise.
Furthermore, this Ca\(^{2+}\)-dependent block of the DxS \([\text{Ca}^{2+}]_i\) rise was consistently observed following application of several chemicals including Pyrazole 3 (Pyr3, a potent and selective TRPC3 antagonist), flufenamic acid (FFA), capsazepine, NS8593 and norgestimate. Shown in Figure 3.3C (yellow), preincubation with 5 \(\mu\)M Pyr3 (5-butyl-1\(H\)-pyrazole-3-carboxylic acid) promoted a persistent elevation in background \([\text{Ca}^{2+}]_i\); and prohibited the expected \(\text{Ca}^{2+}\) influx caused by adding DxS (yellow, \(n = 8\), \(p < 0.0001\)). Likewise, the sustained rise in background \([\text{Ca}^{2+}]_i\); triggered by the addition of 100 \(\mu\)M FFA (2-[[3-(Trifluoromethyl)phenyl] amino] benzoic acid) also abolished the DxS influx (Figure 3.3D, aqua; \(n = 4\), \(p < 0.0001\)). Notably, as observed with ConA, the addition of Pyr3 or FFA late (i.e. after the rise onset, Figure 3.3C & D insets, black), caused an additive \([\text{Ca}^{2+}]_i\); rise. Interestingly, the time courses of these rises were different, suggesting activation of other channels. Together, these findings indicate that the DxS \([\text{Ca}^{2+}]_i\); rise may be 1) modulated by mechanisms involved in store \(\text{Ca}^{2+}\) release and SOCE activation, such as IP\(_3\)R activation or STIM1 binding, or 2) the channel or its signalling components may themselves be inhibited by \([\text{Ca}^{2+}]_i\).

As stock solutions of chemicals, such as Pyr3 and NS8593 (N-[(1R)-1,2,3,4-tetrahydronaphthalen-1-yl]-1\(H\)-1.3-benzodiazol-2-amine), were made using DMSO as the solvent, I confirmed that 1% DMSO did not elevate the background \([\text{Ca}^{2+}]_i\); or alter the DxS rise (Figure 3.3E, black and pink). Notably, when used in experiments, stock solutions were diluted by a factor \(\geq 10^3\), and DMSO was not considered likely to confound the results.

3.3.4  The mechanism is temperature sensitive

Another factor affecting reproducibility was variation in recording temperature. Therefore, to monitor if the temperature remained within physiological range (37 ± 1°C), a Brannan immersion thermometer was placed into the water bath. Significantly, the DxS-induced \(\text{Ca}^{2+}\) rise could not be activated under conditions
where the pre-warm and acquisition temperatures were ≤30°C (Figure 3.2F, light blue; \( n = 7, \ p < 0.0001 \)). Furthermore, maintaining cells on ice raises the background [Ca\(^{2+}\)]\(_i\) compared to cells held at RT and at 37°C. This is depicted in Figure 3.2F (inset) which shows [Ca\(^{2+}\)]\(_i\) in cells, when monitored at 4°C, then RT.

**Figure 3.2** [Ca\(^{2+}\)]\(_i\) and thermal inhibition of the DxS rise

(A) Time courses of DxS [Ca\(^{2+}\)]\(_i\) (blue) with addition of 30 µg/mL ConA at the peak of the DxS rise (\( t = 20 \text{ min; red arrowhead} \)), without DxS but with ConA alone (red with red arrow at \( t = 0 \)), and with DxS (in Ca\(^{2+}\)-free-HBS) and in the presence of 100 µM EGTA (ochre) and subsequent

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addition of ConA at $t = 20$ min. (B) Raw ratio of $\Delta F/F_0$ given for normal DxS rise (blue) and when pre-incubated with ConA for 15 min (red arrow, brown trace). (C) Time courses of DxS ($[Ca^{2+}]$ under normal conditions (blue) when DxS was added at $t = 0$ (grey arrowhead) and when preincubated with Pyr3 (yellow arrow, trace) for 5 min prior to the DxS addition. Inset: Pyr3 added at the onset of the DxS Ca rise ($t = 5$ min, black). (D) Raw ratio of $\Delta F/F_0$ given for normal DxS ($[Ca^{2+}]$ under control conditions (blue) with DxS added at $t = 0$ (grey arrowhead) and when also preincubated with FFA for 5 min (aqua arrow, trace). Inset: FFA added during the DxS rise at $t = 8$ min (black). (E) Time course of DxS induces $[Ca^{2+}]$ under control conditions (blue) and in the presence of 1% DMSO (purple). Black trace indicates $[Ca^{2+}]$ after addition of only DMSO at $t = 0$ min. (F) Time courses of DxS ($[Ca^{2+}]$ under normal conditions (blue) when DxS was added at $t = 0$; when the sample was prewarmed and acquired at 30°C (light blue), and when the temperature was raised to 40°C at $t = 8$ min (black arrow, trace). Inset: Time course $[Ca^{2+}]_i$, in cells monitored at 4°C, then RT from $t = 5$ min, then 37°C from $t = 11$ min then 4°C from $t = 17$ min (red).

(removed from ice bath at $t = 5$ min). At $t = 11$ min, the sample was placed in the heating jacket and warmed to 37°C and finally returned to the ice bath at $t = 17$ min. This observation suggests that the ion channel(s) and/or the signalling cascade involved have a complex temperature sensitivity.

3.3.5 **SOCE via STIM/Orai channel activation is not involved**

The classically described SOCE channels, comprising STIM/Orai subunits, are expressed in DP thymocytes (Gwack et al. 2008). Of the three Orai isoforms, Orai2 mRNA has the highest expression (see Appendix 6; p.357). Notably, the ability of Orai2 to form heteromeric Orai1/Orai2 channels may result in alteration of the Orai channel characteristics during early positive selection reducing their activation (Bertin et al. 2014, Vaeth et al. 2017, Vig & Kinet 2009).

Pharmacologically induced emptying of $[Ca^{2+}]_{ER}$ by the inhibition of the SERCA pump with cyclopiazonic acid (CPA) is a common *in vitro* method used to activate STIM/Orai channels. In thymocytes, the depletion of $[Ca^{2+}]_{ER}$ following the addition of 20 µM CPA rapidly activates them. Using the low affinity dye Mag-fluo-4 to monitor $[Ca^{2+}]_{ER}$ (Diercks et al. 2017, Gerasimenko et al. 2014), I provide evidence of a considerable ER Ca$^{2+}$ leak in these cells. As shown in Figure
3.3A, the addition of 20 µM CPA at $t = 0$ min resulted in a small transient increase immediately followed by a large decrease in Mag-fluo-4 fluorescence (black), distinctly different to the maintained fluorescence in the control sample (purple). This suggests that when the SERCA pump is blocked, i.e. when replenishment of the store is prevented, the fast and large drop in $[\text{Ca}^{2+}]_{\text{ER}}$ can only be explained by a persistent $\text{Ca}^{2+}$ leak.

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)

**Figure 3.3** The DxS $[\text{Ca}^{2+}]_{\text{i}}$ rise is independent of store operated STIM/Orai activation.

(A) Mag-fluo-4 fluorescence during control (pink) and after the addition of CPA to the sample at $t = 0$ (black) suspended in a nominally $\text{Ca}^{2+}$ free solution. (B) Normalised indo-1 fluorescence when SOCE channels were activated by 20 µM CPA (black) and after the addition of 1 µM YM58483 (red) to block Orai channels, isolating $\text{Ca}^{2+}$ release from the ER. The calculated difference between black and red indicates the $\text{Ca}^{2+}$ influx through Orai channels (grey). (C) Time course of
the DxS induced Ca\(^{2+}\) rise with or without YM58483 (red arrow/aqua trace and blue, respectively). Added for comparison is the SOCE component from E (grey).

Because the usage of Mag-fluo-4 with a \(K_d\) of 20–25 µM for Ca\(^{2+}\) precludes the monitoring of the much smaller [Ca\(^{2+}\)]\(_i\) changes with DxS, the involvement of SOCE was then tested with indo-1. In Figure 3.3B, the addition of 20 µM CPA to the sample at \(t = 0\) caused a [Ca\(^{2+}\)]\(_i\) increase with much shorter latency, faster rise and larger amplitude. It reached a peak after ~4 min followed by a small decay afterwards (black). This rise was very different to that seen with DxS and is likely caused by activation of SOCE channels, perhaps STIM/Orai. YM58483 (also known as BTP2) has been described as an indirect inhibitor of Orai channel activation (Ishikawa et al. 2003, Mercer et al. 2010, Zitt et al. 2004). In Jurkat cells, Ishikawa et al. (2003) found that this chemical had an \(IC_{50}\) of ~150 nM, and importantly, concentrations up to 3 µM did not affect the resting [Ca\(^{2+}\)]\(_i\). I therefore chose 1 µM to test if Orai channels were involved. The results as depicted in Figure 3.3B (red) show that Orai channels could be significantly inhibited after pre-incubation of the sample in 1 µM YM58483; i.e. the Ca\(^{2+}\) rise caused by CPA was curtailed (red). To get an idea as to how much Ca\(^{2+}\) was the result of SOCE, the arithmetic difference between the total Ca\(^{2+}\) rise caused by CPA and after Orai channels were blocked is illustrated in grey. It shows that SOCE is delayed and rises much slower but is still considerably faster than that by DxS. In addition, while these channels were blocked (Figure 3.3C), the addition of DxS still caused a [Ca\(^{2+}\)]\(_i\) rise (light blue), indistinguishable from the case when no YM58483 was present (blue, \(n = 4\), \(p_t = 0.98\)). This line of evidence suggests that SOCE via Orai channels is not involved in the DxS [Ca\(^{2+}\)]\(_i\) rise.

However, these results do not entirely rule out activation of a non-selective cation channel. For example, a number of TRP channels are reportedly modulated by STIM1 and IP\(_3\)R (Albarran et al. 2014, Horinouchi et al. 2012, Jardin et al. 2009,
Yuan et al. 2007). If this were the case, then there should also be a concomitant [Na\(^{+}\)]\(_i\) rise in these cells.

### 3.3.6 Concomitant [Na\(^{+}\)]\(_i\) rise

To investigate whether the addition of DxS also led to a rise in [Na\(^{+}\)]\(_i\), cells were loaded with either 5 µM of the ratiometric Na\(^{+}\)-sensitive dye SBFI-AM or 5 µM ANG-2-AM. Using the latter dye, it is possible to also image Ca\(^{2+}\) simultaneously with 5 µM indo-1-AM.

The addition DxS still evoked the same delayed [Ca\(^{2+}\)]\(_i\) rise (blue; axis on the left) but also caused a [Na\(^{+}\)]\(_i\) rise (green; axis on the right) with very similar time course (Figure 3.4A). Fitting sigmoidal functions to both the Ca\(^{2+}\) and Na\(^{+}\) time courses revealed that the \(t_{\text{half}}\) values of the two rises were not different (10.8 ± 0.2 vs. 10.9 ± 0.2 min; \(n = 50\), \(p_t = 0.14\)), but the rate of the [Na\(^{+}\)]\(_i\) rise was marginally faster (0.19 ± 0.01 vs. 0.21 ± 0.01 min\(^{-1}\), \(p_t < 0.0001\)).

As with Ca\(^{2+}\), compared to the other three populations, DP thymocytes showed the largest [Na\(^{+}\)]\(_i\) rise (Figure 3.4B). Moreover, this rise was also only seen around physiological temperature. Like the Ca\(^{2+}\) results, the DxS-induced Na\(^{+}\) rise could not be activated under conditions where the pre-warming and acquisition temperatures were ≤30°C (Figure 3.4C, light green; \(n = 7\), \(p_t < 0.0001\)). As observed for [Ca\(^{2+}\)]\(_i\) (Simon Davis 2015), a much lower DxS concentration (1 µg/mL) was also still sufficient to activate the [Na\(^{+}\)]\(_i\) rise.

I noticed that the background SBFI fluorescence without stimulation decayed slowly with time (Figure 3.4B, black). This is likely caused by inappropriate single wavelength excitation (Borin & Siffert 1990) and perhaps also intracellular acidification associated with use of the HEPES buffer (Cowan & Martin 1995), reducing the emission of SBFI fluorescence (Diarra et al. 2001, Iamshanova et al. 2016). Unlike with SBFI, detection of background ANG-2 fluorescence was stable.
throughout (Figure 3.4D, grey). As for Ca\(^{2+}\) imaging, there was no significant difference between the relative ANG-2 \(\Delta F/F_0\) values obtained from samples measured early or late (Figure 3.4C; \(n = 19\), 0.336 ± 0.038 vs. 0.324 ± 0.039, \(p = 0.82\)).

To get an estimate of the [Na\(^{+}\)] accumulation, I calibrated ANG-2 as illustrated in Figure 2.2A, and found a value of 37 ± 4 mM for its \(K_d\), comparable to that reported by Iamshanova et al. (2016; 34 mM). Using this and an estimated a background [Na\(^{+}\)] value of 9.4 mM (Harootunian et al. 1989) the DxS induced Na\(^{+}\)
are joined by a line. (D) Time courses of DxS [Na\(^+\)]\(_i\) under normal conditions (green) when DxS was added at \(t = 0\) and when the sample was both prewarmed and acquired at 30°C (light green). No stimulus control in grey.

rise was estimated to be 20 ± 3.4 mM. Since this value was surprisingly large, I wanted to obtain an independent estimate based on the [Na\(^+\)]\(_i\) rise caused by ConA (Figure 2.2B), which had been measured to be ~6 mM (Segel et al. 1979). As the increase in fluorescence of ANG-2 is 3-fold higher in DxS than in ConA this means that DxS raised [Na\(^+\)]\(_i\) by 19.8 ± 5.0 mM (\(n = 5\)), consistent with my previous estimate. This indicates that within the about 10 min of the Na\(^+\) rise, there is a large concentration change most likely associated with a significant depolarisation and conductance change.

Like the [Ca\(^{2+}\)]\(_i\) rise, the [Na\(^+\)]\(_i\) rise did not occur when the sample was prewarmed and acquired at 30 rather than 37°C (Figure 3.4D, light green; \(n = 7\), \(p < 0.0001\)). These data show that concomitant with the [Ca\(^{2+}\)]\(_i\) rise, there is a [Na\(^+\)]\(_i\) rise with a similar time course and properties. These two observations are consistent with the idea that the underlying influx may be via a non-selective cation channel (one channel hypothesis) or a set of two channels with similar activation properties (two channel hypothesis).

Since the Na\(^+\) influx could be the result of Ca\(^{2+}\) clearance via the NCX, an important consideration was to check if perhaps the [Ca\(^{2+}\)]\(_i\) rise was dependent on a Na\(^+\) influx.
3.4 Relationship between $[\text{Ca}^{2+}]_i$ and $[\text{Na}^+]_i$

3.4.1 $[\text{Ca}^{2+}]_i$ rise is partially dependent on $\text{Na}^+$ influx

Because $\text{Ca}^{2+}$ homeostasis is not independent of that of $\text{Na}^+$, particularly under conditions when NCX is involved, the relationship between the $\text{Na}^+$ rise and $\text{Ca}^{2+}$ rise was investigated using both $\text{Na}^+$ substitution and the pharmacological block of NCX.

When $[\text{Na}^+]_o$ was replaced with the organic cation NMDG, the resultant DxS $[\text{Ca}^{2+}]_i$ rise was $\sim$45\% smaller than that during control (Figure 3.5A & B, grey; $n = 7$, $p < 0.001$). Fitting a sigmoid curve to each trace (red), I found the half rise and rate values were not significantly different ($n = 3$; $p_t = 0.87$ and 0.95, respectively). This data suggests that there is likely a role for NCX in reverse mode by transporting 3 $\text{Na}^+$ ions out and adding one to the $[\text{Ca}^{2+}]_i$ influx, most likely as the rise begins to plateau.

However, it needs to be pointed out that the cation NMDG may also alter the charge interaction of DxS with the thymocyte membrane. To rule this possibility out, NCX was pharmacologically blocked. Both YM244769 ($\text{IC}_{50} \sim 70$ nM) and SN-6 ($\text{IC}_{50} \sim 3$ µM) selectively inhibit all NCX isoforms by preferentially blocking the reverse mode (Iwamoto et al. 2004, Iwamoto et al. 2007).

When added to the cell suspension without DxS, neither 0.5 µM YM244769 nor 3 µM SN-6 significantly affected background $[\text{Ca}^{2+}]_i$ over time ($n = 11$; $p_t = 0.1$). Significantly, when either of these NCX inhibitors were added prior to DxS, the peak amplitude $[\text{Ca}^{2+}]_i$ rise was reduced by $\sim 45\%$ ($n = 6$, $p_t < 0.001$), as shown in Figure 3.5G. This reduction was comparable to that seen with NMDG ($p_t = 0.9$), suggesting that altered charge screening near the membrane surface is not a major confounding factor. The effect on the peak amplitude of the $[\text{Na}^+]_i$ rise was
highly variable, but overall not significantly different to the control rise (Figure 3.5C; \( n = 8, p_t = 0.4 \)).

![Figure 3.5](image)

**Figure 3.5 The \( \text{Ca}^{2+} \) rise is not dependent upon \( \text{Na}^+ \) entry**

(A) Time course of the normalised \( [\text{Ca}^{2+}]_i \) rise after Dxs (blue) and when \( [\text{Na}^+]_o \) was replaced by NMDG (grey), fitted sigmoid curve (red). (B) Peak amplitude of Dxs \( [\text{Ca}^{2+}]_i \) rise when \( [\text{Na}^+]_o \) was replaced by NMDG normalised to control (dashed line). (C) Box-and-whisker plots of relative peak amplitudes of \( [\text{Ca}^{2+}]_i \) (blue) and \( [\text{Na}^+]_i \) (green) in the presence of SN6 or YM244769 with the control value indicated by the dash. *** for \( p < 0.001 \).

This finding was unexpected as blocking NCX reverse mode should have resulted in \( \text{Na}^+ \) accumulation over time. It is likely explained by the fact that the exchange by NCX is much smaller than the change caused by \( \text{Na}^+ \) influx. These data lend support to the idea that about 40% of the \( \text{Ca}^{2+} \) amplitude is caused by reverse mode activity of NCX. Having established that the \( \text{Ca}^{2+} \) influx was dependent to some extent on \( \text{Na}^+ \) influx, in the next set of experiments, I tested the converse, namely if the \( [\text{Na}^+]_i \) rise depended on \( \text{Ca}^{2+} \) influx.

### 3.4.2 \( [\text{Na}^+]_i \) rise does not depend on \( \text{Ca}^{2+} \) influx

If the \( [\text{Na}^+]_i \) rise depended on a \( \text{Ca}^{2+} \) influx, chelation of \( [\text{Ca}^{2+}]_o \) with EGTA should have abolished it. As illustrated in Figure 3.6A & B when compared to control (green), the time course and amplitude of the Dxs \( [\text{Na}^+]_i \) rise remained unaltered (ochre; \( n = 10, p_t = 0.96 \)). While this finding is consistent with activation of a non-
selective cation channel (one channel hypothesis), an alternative explanation is that Na\(^+\) rose because of a concomitant activation of a voltage-dependent Na\(^+\) channel (two channel hypothesis). To rule this possibility out, Nav were blocked with various concentrations of tetrodotoxin (TTX), a pan-Nav blocker.

Figure 3.6 DxS [Na\(^+\)]\(_i\) rise does not depend on [Ca\(^{2+}\)]\(_o\)

(A) DxS [Na\(^+\)]\(_i\) rise measured with ANG2 when [Ca\(^{2+}\)]\(_o\) was chelated with 100 µM EGTA (ochre) compared to control DxS [Na\(^+\)]\(_i\) rise (green). No stimulus control in grey. (B) Box-and-whisker plots of peak amplitudes of DxS [Ca\(^{2+}\)]\(_i\) (blue) and [Na\(^+\)]\(_i\) (green) when Ca\(^{2+}\) was chelated extracellularly with EGTA (ochre) relative to that of control (100%, dash). Notably, in the presence of EGTA, [Ca\(^{2+}\)]\(_i\) is below normalised 0. This can be explained by the constitutive Ca\(^{2+}\) leak from stores and active transport of Ca\(^{2+}\) from the cell.

3.4.3 Na\(_v\) channels are not involved in [Na\(^+\)]\(_i\) rise

In 2012, Lo et al proposed that a Na\(_v\)1.5 channel facilitated the Ca\(^{2+}\) influx in positively selecting DP thymocytes and consequently played an essential role in selection, particularly for CD4 SP development. In their experimental protocol, the authors did not monitor [Na\(^+\)]\(_i\). However, they did show that addition of 1 µM TTX significantly reduced the [Ca\(^{2+}\)]\(_i\) rise. Notably, the rise was not completely abolished. This was perhaps because Na\(_v\)1.5 channels are quite resistant to TTX (IC\(_{50}\) ~3 µM, Cribbs et al. 1990, Onkal et al. 2008).

To investigate Nav channel involvement, concentrations of 1, 5 and 10 µM TTX were applied either with DxS (t = 0) or, because TTX sterically impedes Na\(^+\)
permeation through the open channel pore (Lipkind & Fozzard 1994), it was also added after the onset of the [Na\(^+\)] rise (i.e. when channels have started to open; \( t = 7 \) min). The results of such experiments are shown in Figure 3.7A. Here the representative Na\(^+\) time course plots are presented for a set of experiments with and without 1 or 5 \( \mu \)M TTX. In both instances, the time courses more or less overlapped. Whisker plots shown in Figure 3.7B indicate there was no statistically significant difference in either the Dxs [Ca\(^{2+}\)] (\( n = 8, p_t = 0.59 \)) or [Na\(^+\)] rise (\( n = 5, p_t = 0.5 \)) when the TTX was added (combined 1 or 5 \( \mu \)M data). Furthermore, the addition of 10 \( \mu \)M TTX, thought to be sufficient to block TTX-resistant Na\(_V\)1.5 channels (Fraser et al. 2004), also did not significantly alter the Dxs [Ca\(^{2+}\)] rise (\( n = 3, p_t = 0.66 \)). These findings indicate that Nav channel activation was not necessary for the Dxs [Ca\(^{2+}\)] rise and further support an argument for a non-selective cation channel.

**Figure 3.7** Dxs [Na\(^+\)] rise does not depend Nav channels.

(A) Time courses of Dxs induced change in ANG2 fluorescence in the presence of 5 \( \mu \)M (pink, added at \( t = 0 \)) or 1 \( \mu \)M TTX to block Nav channels (black, added at \( t = 7 \) min) against that without (green). (B) Respective box-and-whisker plots of the relative peak amplitudes of [Ca\(^{2+}\)] (blue) and [Na\(^+\)] rise (green) in the presence of TTX, with the control peak value indicated by the dash.
### Elevated [Mg\(^{2+}\)]\(_o\) blocks both rises

Interestingly, I found both the Dxs Ca\(^{2+}\) and Na\(^+\) rises were reduced by [Mg\(^{2+}\)]\(_o\) in a concentration dependent manner. Shown in Figure 3.8A, compared to control (blue), the addition of 30 mM Mg\(^{2+}\) 1 min before Dxs abolished the [Ca\(^{2+}\)]\(_i\) rise (black, \(n = 3, p < 0.0001\)), while the addition of 10 mM Mg\(^{2+}\) (C, grey) led to a partial reduction. The [Na\(^+\)]\(_i\) rises were similarly affected, though notably the addition of 30 mM Mg\(^{2+}\) (Figure 3.8B, black, \(n = 3, p < 0.001\)) caused a small rise in [Na\(^+\)]\(_i\), perhaps indicative of Mg\(^{2+}\)/Na\(^+\) antiporter activity (Gunther & Vormann 1992). This data indicates that there is likely a Mg\(^{2+}\)-sensitive channel involved in the cation rise.

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#### Figure 3.8 Increased [Mg\(^{2+}\)]\(_o\) reduces the Ca\(^{2+}\) and Na\(^+\) influx

(A) Time course of the Dxs [Ca\(^{2+}\)]\(_i\) rise in control (blue) and when 30 mM Mg\(^{2+}\) were added at \(t = -1\) min (black arrow/trace). (B) As in (A) but for [Na\(^+\)]\(_i\) with the control green. (C and D) Same as in (A) and (B) but for 10 mM Mg\(^{2+}\) (grey).
3.4.5 Elevated background [Ca^{2+}] also abolishes the Na\(^+\) influx

As described in 3.3.3, elevated background [Ca^{2+}] prohibited the DxS [Ca^{2+}] rise. I therefore checked if such a rise also occluded the [Na\(^+\)] rise. To elevate background [Ca^{2+}], I used ConA. Unlike the relatively fast-onset and transient [Ca^{2+}] rise observed by the addition of 30 μg/ml ConA on its own (see Figure 3.2A), using ANG-2 revealed a small gradual [Na\(^+\)] rise (Figure 3.9A, red). This is likely explained by NCX activity as a consequence of the concomitant Ca\(^{2+}\) load. As in Figure 3.2A, ConA was applied during the plateau phase at \(t = 20\) min (Figure 3.9A, red arrowhead), but in contrast to the Ca\(^{2+}\) response, there was no small transient increase with [Na\(^+\)] (green). Even with [Ca\(^{2+}\)]\(_o\) chelated with 100 μM EGTA, no change was seen either (ochre).

![Figure 3.9](image)

**Figure 3.9** DxS [Na\(^+\)] is abolished by increased background [Ca\(^{2+}\)].

(A) Time courses DxS [Na\(^+\)] under control (green) and when extracellular Ca\(^{2+}\) was chelated with EGTA (ochre). At the time of the red arrowhead, ConA was added. Time course of [Na\(^+\)], when ConA but no DxS was added at \(t=0\) min (red). (B) Time course of raw \(\Delta F/F_0\) when DxS was added at \(t = 0\) min during control and after pre-incubation with ConA for 15 min (brown).

As previously observed when monitoring [Ca\(^{2+}\)] (Figure 3.2B), I found preincubation with ConA abolished any further DxS induced [Na\(^+\)] rise (Figure 3.9B, brown). Similarly, in this plot I have not normalised to the control \(\Delta F/F_0\) in order to make an elevated background [Na\(^+\)] visible. The control [Na\(^+\)] rise is shown in green, and when the sample was pre-incubated for 15 min in ConA.
(brown). This time course indicates that after pre-incubation, not only \([\text{Ca}^{2+}]_i\) but also background \([\text{Na}^+]_i\) was elevated. Notable though is the fact that there is no \([\text{Na}^+]_i\) rise following addition of DxS (Figure 3.9B; brown). This was the case in all such experiments \((n = 7, p < 0.0001)\). This indicates that activation of the non-specific cationic influx is prevented by a prior rise in background \([\text{Ca}^{2+}]_i\) and/or \([\text{Na}^+]_i\).

The findings so far suggest that the addition of DxS to DP thymocytes activates a non-selective cation influx. In addition, a rise in background \([\text{Ca}^{2+}]_i\) by \(\sim 60\) nM and/or \([\text{Na}^+]_i\) prevents its activation. In the presence of a small and sustained elevation in background \([\text{Ca}^{2+}]_i\), this apparent inhibition raises the question if the block is due solely to the increased \([\text{Ca}^{2+}]_i\) and/or \([\text{Na}^+]_i\) or if the activation of the influx is negatively modulated by changes in STIM1 or IP3R, that are associated with \([\text{Ca}^{2+}]_\text{ER}\) release.

The fact that the influx occurs via a non-specific ion channel would support many TRP channels as potential candidates. Since most TRP channels are modulated by intracellular signalling cascades (reviewed by Numata et al. 2011), it was necessary to investigate the involvement of cell signalling prior to the \([\text{Ca}^{2+}]_i\) influx.
3.5 Molecular mechanisms upstream of the cation influx

3.5.1 PLC activation is required

To determine if PLC activity was necessary upstream of the rise activation, edelfosine (1-octadecyl-2-methylglycer-3-phosphorylcholine), an ether lipid analogue, was used to inhibit the activation of all isoforms (Horowitz et al. 2005). Since both PLC-γ1 and -β2 isoforms are moderately expressed in these cells, the precise target remains unclear. The cell suspension was pre-incubated with 1 µM edelfosine for 1 min. As shown in Figure 3.10A & B and summarised in C for \([\text{Ca}^{2+}]_i\) and \([\text{Na}^+]_i\) (blue and green respectively), the addition of edelfosine prior to DxS resulted in a significant inhibition in both the \(\text{Ca}^{2+}\) (pink; \(n = 4, p_t < 0.0001\)) and \(\text{Na}^+\) influx (\(n = 4, p_t < 0.01\)). Furthermore, comparison of the two mean inhibitions revealed that there was no difference between them (\(p_{pt} = 0.4\)). These data suggest that the activation of PLC is critically involved in generating the DxS \(\text{Ca}^{2+}\) and \(\text{Na}^+\) influx. Notably, 30 µM edelfosine (IC\(_{50}\) = 9.6 µM, Powis et al. 1992) caused an extreme rise in \([\text{Ca}^{2+}]_i\) and loss of the indo-1 signal (Figure 3.10A inset).

3.5.2 Not modulated by \([\text{Ca}^{2+}]_\text{ER}\) depleted STIM1 activation or IP₃R

In thymocytes, depletion of \(\text{Ca}^{2+}\) in the ER normally occurs when PLC-γ1 generates IP₃ that then activates IP₃Rs. This in turn can lead to STIM1-dependent activation of SOCE channels. The subsequent \(\text{Ca}^{2+}\) influx then replenishes the \([\text{Ca}^{2+}]_\text{ER}\) via the SERCA pump uptake, altogether homeostatically maintaining \([\text{Ca}^{2+}]_\text{ER}\).

In section 3.4.4, I showed that the DxS activated rise was not due to STIM/Orai or a TRPC3 channel. While STIM1 is generally associated with SOCE, it can also function as a regulatory mechanism in ROCE. For instance, STIM1 regulates translocation of receptor-operated TRPC6 channels to the PM (Albarran et al. 2014), and its association with TRPA1 negatively modulates STIM1/Orai.
interaction (Albarrán et al. 2013). In addition, IP₃R binding on the C terminus of TRPC and TRPV4 channels has been shown to modulate channel activity (Garcia-Elias et al. 2008, Zhang et al. 2001). Therefore, investigation of a potential role for STIM1 and/or IP₃R activation in regulating a second messenger-activated channel was still warranted.

Previously, I have shown that [Ca²⁺]ₑ₍ₑ₎ could be depleted by concurrently blocking both Orai channels and the SERCA pump. This protocol was again used to examine if store release and associated STIM1 activation significantly altered the DxS rise.

Figure 3.10D shows the effect over time of concurrent inhibition of Orai channel by 1 µM YM58483 and the SERCA activity by 20 µM CPA (red). With STIM1-dependent transmembrane Ca²⁺ influx (via Orai channel, activation) and Ca²⁺ sequestration into the ER blocked, [Ca²⁺]ᵢ initially rapidly rose followed by an exponential decay back to background [Ca²⁺]ᵢ and after ~10 min was indistinguishable from baseline [Ca²⁺]ᵢ. I note that there was no concomitant [Na⁺]ᵢ rise. This observation is consistent with the appreciable constitutive Ca²⁺ leak from ER stores mentioned above (section 3.3.5), which depletes [Ca²⁺]ₑ₍ₑ₎ to activate SOCE (Camello et al. 2002, Foyouzi-Youssefi et al. 2000).

Having depleted [Ca²⁺]ₑ₍ₑ₎ and likely induced a conformational change in STIM1, DxS was added in an experiment at t = 0 min. As shown in Figure 3.10D, this still resulted in subsequent rises in Ca²⁺ (light blue) and Na⁺ (light green), which were not different to the normal DxS rises, respectively (n = 6, pᵦ = 0.11; n = 3, pᵦ > 0.07). These rises also had a similar delay of ~5 min. This suggests that both DxS rises remained largely unchanged. I note that within ~6 min of depleting stores, [Ca²⁺]ᵢ had returned to close to the background level, which likely “re-enabled” the subsequent rises. This suggests that most likely increased background [Ca²⁺]ᵢ is solely responsible for blocking channel activation or the preceding signalling
cascade. These data provide evidence that both rises are not significantly modulated by activation of STIM1 by [Ca\textsuperscript{2+}]\textsubscript{ER} depletion. However, a role for thermally activated STIM1 (Liu et al. 2019, Xiao et al. 2011) in regulation of this cation rise cannot be discounted and remains to be determined.

Figure 3.10 Activation of PLC-γ1 is required, but not SOCE

(A) Time course plots of the DxS [Ca\textsuperscript{2+}]\textsubscript{i} rise control (blue) and when incubated with edelfosine (pink arrow/trace). Inset: 30 μM edelfosine added at \( t = 0 \) min caused massive rise and then loss of indo-1 emission detection. (B) As in (A) but monitoring the DxS [Na\textsuperscript{+}]\textsubscript{i} rise; control (green) and pre-treated with edelfosine (pink). (C) Box-and-whisker plot (pink) summarising the effect of added edelfosine on the DxS [Ca\textsuperscript{2+}]\textsubscript{i} rise and DxS [Na\textsuperscript{+}]\textsubscript{i} rise. (D) Time course plots of the DxS [Ca\textsuperscript{2+}]\textsubscript{i} rise when DxS was added at \( t = 0 \) min (grey arrowhead). Control DxS [Ca\textsuperscript{2+}]\textsubscript{i} rise (blue) and when Orai channels were inhibited (1 μM YM58483, black arrow) and the ER store depleted (CPA 20 μM, red arrow, aqua trace). Appended in this plot is the DxS [Na\textsuperscript{+}]\textsubscript{i} rise when treated with YM58483 + CPA (black & red arrows, respectively) before adding DxS at \( t = 0 \) min (grey arrowhead; light green). Also shown is a time course (red) of the [Ca\textsuperscript{2+}]\textsubscript{i} rise when YM58483 and CPA were added (black & red arrow, respectively).
To demonstrate if IP₃R activation were involved, the IP₃R antagonist xestospongian C (XestC) was used. Initially identified as a selective inhibitor of IP₃Rs with an IC₅₀ of 358 nM (Gafni et al. 1997), its pharmacology is debated as to its target(s). While it has been argued that XestC inhibits the SERCA pump rather than IP₃R (Castonguay & Robitaille 2002, De Smet et al. 1999, Solovyova et al. 2002), Ta et al. (2006) concluded, based on data acquired by specifically monitoring [Ca²⁺]ᵅ, that XestC inhibited IP₃R activity but did not inhibit the SERCA pump. Notably, inhibition of IP₃R by XestC slowly increases with time due to the slow on-rate (De Smet et al. 1999). Therefore, samples were first pre-incubated with 2 µM XestC at 37°C for 10 min. To control for the longer incubation time, the control samples were likewise pre-warmed.

The addition of 2 µM XestC to thymocytes by itself did not produce a [Ca²⁺]ᵅ rise (Figure 3.11B, black; n = 4, pᵦ = 0.99), suggesting that it did not significantly inhibit the SERCA pump. In samples pre-incubated with XestC prior to DxS (n = 4), the characteristics of the rises, including the [Ca²⁺]ᵅ and [Na⁺]ᵅ peak amplitudes (shown in Figure 3.11A), were not significantly different compared to the normalised DxS control (Ca²⁺: pᵦ = 0.6; Na⁺: pᵦ = 0.3). This result may indicate that IP₃R activation was not significantly involved in both rises. An alternative and simpler explanation is that XestC failed to inhibit IP₃R.

To rule this possibility out, a large Ca²⁺ rise was evoked by norgestimate. In thymocytes, the addition of 15 µM norgestimate resulted in an immediate Ca²⁺ rise consistent with SOCE (Figure 3.11B, grey). Reportedly, this occurs via activation of a steroid sensitive receptor which in turn activates a Src tyrosine kinase signalling cascade to stimulate PLC-γ1 (Boonyaratanakornkit et al. 2001). To provide evidence that this was the case, I used PP2 (1-tert-butyl-3-(4-chlorophenyl)pyrazolo[4,5-e]pyrimidin-4-amine), a pan-Src kinase inhibitor (Hanke et al. 1996, Irie et al. 1998). Pre-incubation for 10 min at 37°C with 300 nM
PP2 largely abolished the rise seen with norgestimate (Figure 3.11B, pink), lending support to the idea that norgestimate acted via Src kinase to stimulate PLC-γ1. To identify the contribution of Orai channels to the Ca\(^{2+}\) influx, the addition of 1 μM YM58483 together with norgestimate inhibited the sustained rise, whilst leaving a transient rise (red). The IP\(_3\)R contribution was revealed when a sample exposed to the same blockers was pre-incubated with XestC (ochre). Together, these results suggest that 2 μM XestC was capable of blocking IP\(_3\)R, albeit not completely, and consequently rules the simple explanation out.

Furthermore, they support the argument that the DxS rises are dependent upon PLC-γ1 activation but do not require IP\(_3\)R activation nor modulation by store Ca\(^{2+}\) depletion activation of STIM1.

![Figure 3.11 Activation of IP\(_3\)R not required](image)

(A) Box-and-whisker plots (grey) summarising the effect of XestC on the DxS [Ca\(^{2+}\)]\(_i\) (blue) and [Na\(^{+}\)]\(_i\) (green) rise amplitude. The dashed line at 100%, denotes the normalised DxS peak amplitude. (B) Time course plots comparing the [Ca\(^{2+}\)]\(_i\) influx from store release with and without SOCE channel activation following the addition of norgestimate (grey arrow/trace) and when also treated with YM58483 (black arrow, red). Time course of [Ca\(^{2+}\)]\(_i\) following incubation with XestC prior to addition of YM58483 with norgestimate (ochre) and when pre-incubated with PP2 prior to adding norgestimate (grey arrow, pink). Black trace shows the effect of 2 μM XestC added at \(t = 0\) min on [Ca\(^{2+}\)].
Having looked so far at a few mechanisms upstream of the non-specific cation influx, in the following I would like to turn the focus around to investigate mechanisms downstream of DxS binding to surface molecules. As mentioned previously, DxS potentially interacts with multiple targets on the surface of DP thymocytes. Consequently, more than one signalling pathway may turn on.

### 3.5.3 Role for CD8β

Recent evidence suggests that DxS competitively binds to the CD8 coreceptor, particularly the CD8β chain, at a heparan sulfate binding region. Comparing the response in DP thymocytes obtained from CD8β−/− with that of WT mice, Simon Davis (2015) demonstrated that the interaction of DxS with CD8β was crucial for the initiation of the signalling pathway that resulted in the sustained $[\text{Ca}^{2+}]_{i}$ rise.

I confirmed these findings for $\text{Ca}^{2+}$ under the conditions used in this thesis and extended them to include Na+ imaging. Consistent with his findings, not only the DxS $[\text{Ca}^{2+}]_{i}$ was strongly inhibited, but also the $[\text{Na}^{+}]_{i}$ rise was much reduced in cells from CD8β−/− mice (Figure 3.12A & B, red). In this data set and similar to the reduction observed for $\text{Ca}^{2+}$ ($n = 6, p_t < 0.0001$), the mean Na+ rise was $23.7 \pm 3.2\%$ of that observed in WT litter mates ($n = 3, p_t < 0.0001$). However, it is interesting to note that while the DxS $[\text{Ca}^{2+}]_{i}$ and $[\text{Na}^{+}]_{i}$ rises were both much reduced, neither was completely abolished (Figure 3.12C, red boxes; $n = 6, p_t < 0.0001$ and $n = 4, p_t < 0.0001$, respectively). This suggests that DxS likely cross-linked (an)other target(s) on the PM, sufficient to partially activate both influxes.

Furthermore, restriction of the rises to the DP population could be explained by the low level of CD8 coreceptor sialylation (Simon Davis 2015). As thymocytes differentiate to become mature CD8 T cells, the stabilisation of TCR–MHC-I interaction by CD8–MHC-I binding is reduced by increased sialylation of the CD8β coreceptor (Moody et al. 2003). This sialylation also appears to prohibit
activation of the signalling cascade initiated by DxS binding as reducing CD8β sialylation, by prior treatment of mature T cells with neuraminidase (type II from V. cholera), re-established the DxS [Ca^{2+}] rise in CD8 but not CD4 T cells (Simon Davis 2015).

I therefore checked if cleavage of sialic acid by pre-incubation with neuraminidase for 1 h not only re-established the [Ca^{2+}] rise in mature peripheral CD8 lymphocytes, but if this rise was also accompanied by a [Na^+] rise. This is illustrated in Figure 3.12E, where in this case, peripheral T cells when exposed to DxS do not show a [Na^+] rise (olive). But when pre-incubated with neuraminidase, DxS was capable of evoking a similar rise in mature CD8 (aqua) but not CD4 T cells (data not shown). These results confirm that CD8β sialylation is a critical repressor of downstream signalling.

As the DxS was not abolished in CD8β−/− thymocytes (Figure 3.12A & B), I next checked the involvement of LFA-1. Shown in Figure 1.3A (Kim et al. 2009a), activation of this integrin has been shown to evoke a SOCE independent Ca^{2+} influx in conditions of sub-optimal TCR activation.

3.5.4 Role of LFA-1

Notably, this cell adhesion molecule reportedly plays a crucial role in the development of CD8 but not CD4 lineage cells (Revilla et al. 1997). Therefore, the contribution of LFA-1 to the DxS rises was investigated using thymocytes prepared from mice either deficient in the CD11a chain of LFA-1 or WT littermates.
Figure 3.12 CD8 and LFA-1 are required.

(A) Dxs [Ca^{2+}] rises in cells from a WT (blue) and CD8β−/− mouse (red). (B) Same as in (A) but for [Na^+]i. (C) Box-and-whisker plots for Dxs [Ca^{2+}] rise (blue) and the Dxs [Na^+]i (green) in CD8β−/− (red) and LFA-1 deficient cells (grey), with the control Dxs peak amplitude indicated by the grey dashed line. **** p < 0.0001 (D) Time course of Dxs [Na^+]i rise normalised for the peak amplitude in DP thymocytes (green), mature peripheral CD8 T cells (olive), and mature peripheral CD8 T cells pre-treated with neuraminidase (aqua). (E) Time course of Dxs [Ca^{2+}] rises in cells from a WT (blue) and LFA-1−/− mouse (grey). (F) Same as in (E) but for [Na^+]i.
The respective time courses depicted in Figure 3.12E & F (grey), show a large reduction of the rises, comparable to the findings for CD8β⁻/⁻ thymocytes (A & B, red). The peak amplitude of DxS [Ca²⁺]i and [Na⁺]i rises from CD11a-deficient mice were reduced by 69.6 ± 6.5 and 76.9 ± 7.2%, respectively (Figure 3.12C, grey boxes; n = 5, pt < 0.0001). Interestingly, this reduction was not different to the one seen with CD8β⁻/⁻ thymocytes (pt = 0.7 and 0.53, respectively).

However, there is an interesting difference between the CD8β⁻/⁻ and LFA-1⁻/⁻ thymocytes. With LFA-1 deficiency (grey), i.e. when CD8 including CD8β signalling remains intact, there is a slow linear rise to the same peak amplitudes for both Ca²⁺ and Na⁺. This may indicate that this linear rise is caused by the DxS-CD8β interaction. In contrast, with CD8β deficiency (red), i.e. with LFA-1 intact, the rise is supralinear and occurs with a delay, suggesting that LFA-1 may provide a supralinear amplification upon the signalling provided by CD8β.

Both lines of experiment indicate that CD8β and LFA-1 are critical receptor components in the signalling leading up to the two rises. They may suggest that for a full rise, DxS likely interacts with both surface proteins. These observations point to the co-activation of signalling steps downstream of CD8 and LFA-1 binding.
3.5.5 Involvement of Src kinase

It is hypothesised that the signal transduction initiated by DxS binding to the CD8β coreceptor results in the activation of Lck, which belongs to the family of Src kinases. It is highly expressed in DP thymocytes (Figure 3.13A) and importantly, in these cells it is associated with the cytosolic C-terminal of the CD4 and to a lesser extent CD8 coreceptors (Wiest et al. 1993). This sequestration of Lck to the coreceptors ensures that during the selection process, TCR signal activation remains specific to MHC interactions (Van Laethem et al. 2007). However independent of MHC interaction, antibody cross-linking of surface CD8 receptors has been shown to increase tyrosine phosphorylation of Lck (Irie et al. 1998, Veillette et al. 1989). Furthermore, as stabilisation of the CD8–MHC-I interaction is enhanced by Lck-dependent activation of LFA-1 (Lepesant et al. 1990, Morgan et al. 2001), I checked if this Src kinase was involved downstream of DxS binding using the pan-Src kinase inhibitor PP2.

To inhibit Src kinase activity, the thymocytes were incubated at 37°C for 15 min with 100 nM PP2 (IC₅₀ = 4 & 5 nM for Lck and Fyn, respectively; Hanke et al. 1996). To control for the longer incubation, the control sample was equally pre-warmed. Such an experiment is illustrated in Figure 3.13B & C (black). Notably, when Src kinase activity was blocked, both the Ca²⁺ and Na⁺ rises were completely abolished (n = 5, p < 0.0001 in both cases). This indicates that Src kinase (most likely Lck) activation is critical in the signalling downstream of DxS binding. It suggests that tyrosine phosphorylation is essential step.
Figure 3.13 Involvement of Src kinase.

(A) Lck mRNA expression in T lymphocytes (probe set 1439146_s_at). (B) Time course of DxS [Ca^{2+}] rise in control (blue) and after pre-incubation in PP2 (black). (C) Same as in (B), but for [Na^+].

3.5.6 Channel activity may be modulated by PKC and PKA

In addition to Src kinases, in thymocytes, PKC and PKA both play important roles in thymocyte selection and apoptosis (Moen et al. 2017, Ohoka et al. 1996, Zambon et al. 2011). These two important effector kinases are activated by DAG (PKCα, -β, -δ, -ε, -γ, η and θ isoforms) and cyclic adenosine monophosphate (cAMP), respectively. They target a broad repertoire of proteins, and notably the activity of a number of ion channels and the PMCA pump has been shown to be enhanced by PKC and/or PKA phosphorylation (reviewed by Balasubramanyam & Gardner 1995, Mandadi et al. 2011, Venkatachalam et al. 2003).
3.5.6.1 PKC activation likely enhances the signalling cascade

In DP thymocytes, the Ca\textsuperscript{2+}-dependent isoforms PKC\textalpha, and PKC\textbeta, and the Ca\textsuperscript{2+}-independent isoforms, PKC\textdelta, -\eta, and -\theta, are well-expressed and, have roles in modulating signalling pathways that determine selection, apoptosis and differentiation (reviewed by Morley et al. 2008, Ohoka et al. 1996, Pfeifhofer-Obermair et al. 2012, Simon et al. 2000). Therefore, I used the broad-spectrum PKC inhibitor Gö6983 \((3\text{-}[1\text{-}[3\text{-}(dimethylamino)propyl]-5\text{-}methoxy-1\text{H}-\text{indol}-3\text{-}yl]-4\text{-}\text{(1H}\text{-}\text{indol}-3\text{-}yl)-1\text{H}-\text{pyrrole}-2,5\text{-}dione)\) to check if PKC might crucially modulate the DxS [Ca\textsuperscript{2+}]\text{\textasciitilde} rise.

Gö6983 is a potent inhibitor of PKC\textalpha, -\beta, -\gamma, -\delta isoforms with IC\textsubscript{50} values of 7, 7, 6, 10, and 60, respectively (Gschwendt et al. 1996). I found that the addition of 0.1 \textmu M Gö6983 prior to DxS, reduced the peak amplitude of the DxS [Ca\textsuperscript{2+}]\text{\textasciitilde} and [Na\textsuperscript{+}]\text{\textasciitilde} rises by 35.8 \pm 2.8\% and 34.8 \pm 5.2\%, respectively, compared to the normalised DxS rise value, represented by the dashed line in Figure 3.14A \((n = 3, p = 0.015\text{ and } n = 3, p = 0.02,\text{ respectively})\). These results indicate likely enhancement of the signalling cascade by PKC phosphorylation of target molecules.

3.5.6.2 PKA also enhances the cation influx

I next checked the involvement of PKA in the signalling cascade. In DP thymocytes, the cAMP/PKA pathway is reported to mediate apoptosis (Zambon et al. 2011) and in T cells, it has been shown to promote negative regulation of Lck signalling, by way of phosphorylation of C-terminal Src kinase (Csk) which in turn phosphorylates and inhibits Lck (reviewed by Linden & Cekic 2012, Wehbi & Taskén 2016). Having found that activation of Src kinase (most likely Lck) is important in the transduction of the DxS induced signalling cascade, I investigated the role of PKA.
Specifically, I used 500 nM H-89 (N-2-[3-(4-bromophenyl)prop-2-enylamino] ethyl isoquinoline-5-sulfonamide) to inhibit PKA activation (IC₅₀ = 135 nM; Davies et al. 2000). Importantly, as shown in (Figure 3.14B, red boxes), this concentration did not affect resting [Ca²⁺]ᵢ (n = 4, pᵣ = 0.24) or [Na⁺]ᵢ (n = 5, pᵣ = 0.53). Shown in Figure 3.14B (grey boxes) and C & D (grey traces), preincubating cells for 15 min with H-89 significantly reduced the amplitude of the DxS [Ca²⁺]ᵢ rise (n = 4, pᵣ = 0.001) and [Na⁺]ᵢ rise (n = 4, pᵣ = 0.006). These data suggest that PKA has a role in promoting the DxS cation influx mechanism.

Figure 3.14 PKC and PKA activity likely enhance the Ca²⁺ and Na⁺ influx

(A) Box-and-whisker plots of relative peak amplitudes of [Ca²⁺]ᵢ (blue) and [Na⁺]ᵢ (green) in the presence of Gö6983 with the control value indicated by the grey dash, * for p <0.05. (B) As in (A) but in the presence of H-89 (grey boxes), *** for p <0.001 ** for p<0.01. The red boxes show H-89 on its own did not significantly alter resting [Ca²⁺]ᵢ and [Na⁺]ᵢ compared to untreated cells, indicated by the red dash. (C) Time course of DxS [Ca²⁺]ᵢ rise in control (blue) and after pre-incubation in H-89 (grey). (D) Same as in (C), but for [Na⁺]ᵢ. (E) Box-and-whisker plots of relative peak amplitudes of [Ca²⁺]ᵢ (blue) and [Na⁺]ᵢ (green) in the presence of caffeine (black boxes) which are not significantly different from the control value indicated by the grey dash. Red boxes show
the addition of caffeine did not significantly change resting $[Ca^{2+}]_i$ and $[Na^+]_i$ compared to untreated cells, indicated by the red dash.

To further explore the effect of PKA on the Dxs induced cation influx, I used 400 µM caffeine to inhibit phosphodiesterase degradation of cAMP and hence augment the concentration of PKA. Shown in Figure 3.14E (red boxes), 500 µM caffeine did not significantly change resting $[Ca^{2+}]_i$ ($n = 3$, $p_t = 0.12$) or $[Na^+]_i$ ($n = 3$, $p_t = 0.94$). Also shown in this box-and-whisker plot, the addition of caffeine 5 min prior to Dxs (black boxes) caused no significant increase in the peak amplitude of the cation rises ($Ca^{2+}$: $p_t = 0.1$; $Na^+$: $p_t = 0.15$). Notably, there was moderate variation in the data recorded from four experiments.

Finding that PKA and PKC reduced, but did not abolish the Dxs rises, I next investigated the role of PI3K. This kinase has a crucial role in thymocyte transition from the immediate single positive to the preselection DP stage (Xue et al. 2008).

**3.5.7 The cation influx requires PI3K activation**

Recruited to the TCR by CD3ζ and Lck, PI3Kδ activation downstream of Lck signalling increases the production of phosphatidylinositol (3,4,5)-tris-phosphate (PIP3; Okkenhaug & Vanhaesebroeck 2003, Sánchez-Martín et al. 2004). In response to the PIP3 increase, the interleukin-2 tyrosine kinase (Itk) is recruited to the PM where it interacts with the adaptor proteins LAT, SLP-76 and the enzyme PLC-γ1 (Min et al. 2009), the latter of which is critical for the cation influx.

To test if PI3K activation was involved, LY294002 was used to inhibit its activation ($IC_{50} = 10$ µM; Davies et al. 2000). Specifically, the cell suspensions were pre-incubated with 50 µM LY294002 for 15 min at 37°C. Titration experiments found that at this concentration the Dxs rises were optimally inhibited whilst having negligible effect on the resting $[Ca^{2+}]_i$ and $[Na^+]_i$. As shown in Figure 3.15A & B, when exposed to Dxs and compared to control (blue and green), there was a large
reduction in both cation influxes (black), on average by \(72 \pm 4\%\) for \([Ca^{2+}]_i\) \((n = 6; \ p_t < 0.0001)\) and \(46 \pm 4\%\) for \([Na^+]_i\) \((n = 8; \ p_t < 0.001)\). Notably, the effect of inhibiting PI3K resulted in time courses comparable to those observed in the CD8\(\beta^+\) thymocytes (Figure 3.12A & B). Furthermore, as the reduction of the \([Ca^{2+}]_i\) \((n = 5, \ p_t = 0.9)\) and \([Na^+]_i\) rise \((n = 3, \ p_t = 0.09)\) was comparable, it suggests the LY294002 inhibition of PI3K activation was potent.

**Figure 3.15 Inhibition of PI3K reduces the cation influx**

(A) Time courses for Dxs \([Ca^{2+}]_i\) under control conditions (blue), or when prewarmed for 15 min (mauve), and when incubated with LY294002 for 15 min (black) prior to addition of Dxs \((t = 0, \ grey\ arrowhead)\). Inset: Whisker plot showing the effect of 15 min pre-incubation with 1 \(\mu\)M LY294002 on Dxs \([Ca^{2+}]_i\) rise amplitude. (B) Same as in (A) but for \([Na^+]_i\), the 15 min prewarmed control (green) and when preincubated with LY294002 (black). Inset: Whisker plot showing the effect of 15 min pre-incubation with 1 \(\mu\)M LY294002 on Dxs \([Na^+]_i\) rise amplitude. **** for \(p < 0.0001\).

These data suggest a critical role of PI3K in activation of both rises. As PI3K catalyses PIP_3 synthesis by PIP_2 phosphorylation, it is either the PIP_3 availability or the local depletion of PIP_2 that activates the influxes. However, since block of PLC-\(\gamma 1\) also lead to a similar block (see above), it is likely that PIP_2 depletion underpins the two rises.

PI3K activation may lead to increased surface expression of proteins (reviewed by Cayouette & Boulay 2007). Given that it takes ~5 min until the start of the rises,
both membrane insertion and cytoskeletal re-arrangements could be consistent with such a delay. Therefore, the effect of disrupting the actin cytoskeleton on the DxS rises was investigated next.

3.5.8 Cytoskeletal disruption prevents the influx

Inhibition of Lck likely prevents signal transduction via the CD8 coreceptor and indirectly inhibits LFA-1. It appears that LFA-1 activation is dependent upon cytoskeletal rearrangement (Cairo et al. 2006, Morgan et al. 2001, Perez et al. 2003). This involvement with the cytoskeleton downstream of Lck and PI3K raises the hypothesis that F-actin polymerization also plays an essential role in promoting the DxS rises.

In this set of experiments, F-actin polymerization was inhibited using latrunculin B (LatB). In mouse fibroblasts, incubation for 1 h with LatB (~1 µM) has been shown to disrupt microfilaments (Spector et al. 1989). To interfere with actin filaments, thymocytes were incubated with 1 µM LatB for 55 min at RT and then pre-warmed to 37°C for 5 min. The control sample was subjected to the same sequence. Such an experiment is depicted in Figure 3.16A & B. A marked inhibition of the DxS [Ca^{2+}]; (aqua; \( n = 3, p < 0.001 \)) and [Na^+]; rise (light green; \( p < 0.001 \)) was observed (Figure 3.16C). The results of these experiments are consistent with the hypothesis that inhibition of F-actin polymerization with LatB 1 µM impedes the rises.

To rule out if LatB pre-incubation alone markedly increased background [Ca^{2+}]; and [Na^+];, control samples were pre-warmed for 5 min and recording started. 1 µM LatB was added at \( t = 0 \) min and data acquisition continued for a further 5 min (Figure 3.16A & B insets). LatB on its own caused a transient [Na^+]; rise only, which returned to the resting level within 5 min. To check if LatB after 1 hour, changed the background concentrations, the same sample was removed from
recording and left at RT incubation for another 45 min. It was pre-warmed again and acquired on the cytometer for another 5 min (insets). Data was appended to the original recording for comparison of the background $[\text{Ca}^{2+}]_i$ and $[\text{Na}^+]_i$. After 1h, on its own, LatB may have caused a small decrease in resting $[\text{Ca}^{2+}]_i$. However, when compared to a control sample with no LatB added, the change in resting $[\text{Ca}^{2+}]_i$ and $[\text{Na}^+]_i$ after 60 min was not significantly different ($n = 3$, $p_t > 0.05$ for both). These results rule out the possibility that a change in resting $[\text{Ca}^{2+}]_i$ and $[\text{Na}^+]_i$ by LatB caused the disruption of the rises.

Figure 3.16 Disruption of the cytoskeleton inhibits Dxs rises

(A) Dxs $[\text{Ca}^{2+}]_i$ rise in control conditions (blue) and when the sample was incubated with LatB for 60 min prior to the addition of Dxs ($t = 0$ min; aqua). Inset shows $[\text{Ca}^{2+}]_i$, when LatB was added ($t = 0$ min). The effect on $[\text{Ca}^{2+}]_i$ after 1 h was also recorded (black). (B) Same as in (A) but for $[\text{Na}^+]_i$ (control green; LatB light green). (C) Box-and-whisker plot showing the reduction in peak amplitude when incubated with LatB prior to Dxs. The control Dxs rise amplitude is indicated by the black dashed line.
This set of experiments demonstrates that blocking F-actin polymerization resulted in a large reduction of both the [Ca\textsuperscript{2+}]\textsubscript{i} and [Na\textsuperscript{+}]\textsubscript{i} rises. This suggests that downstream of DxS binding, cell signalling includes F-actin polymerization and cytoskeletal changes.

Since the cytoskeleton can shield mechanosensitive elements in the PM from exposure to shear forces, it may be that the cation influxes occur as a consequence of mechanical stimulation. To test this idea, stretch activated cation (SAC) channels were blocked with the tarantula peptide toxin M-thegaphtotoxin-Gr1a (GsMTx4).

Exposure to low micromolar concentrations of GsMTx4 has been shown to specifically inhibit SAC channels (K\textsubscript{c} \approx 600 nM in adult rat astrocytes; Suchyna et al. 2000) by impeding the propagation of tensile forces in the local PM region (Gnanasambandam et al. 2017, Gottlieb et al. 2007, Suchyna et al. 2004). Where the lipid tension is altered in the region of SAC channels, it modifies their gating.

3.5.9 **DxS rises are fully blocked after preincubation with GsMTx4**

To investigate if the addition of DxS leads to altered PM tension, I used 3 \( \mu \)M GsMTx4. It was added to the sample at \( t = -5 \) min to check its effect on the background [Na\textsuperscript{+}] or [Ca\textsuperscript{2+}]. (Figure 3.17A & B; black). Having found it did not increase the resting [Ca\textsuperscript{2+}]; DxS was added at \( t = 0 \) min. Significantly, both rises were fully blocked. This experiment was repeated 4 times and compared to the respective control rises. In all instances, these were completely blocked (\( p < 0.001 \)). This finding indicates that the DxS rises are potently sensitive to GsMTx4 and suggests that mechanically transduced force is involved. This may indicate that, a SAC channel is likely involved.
Figure 3.17 Signal transduction is mechanosensitive

(A) Time courses of DxS $[Ca^{2+}]_i$: control (blue) and when pre-incubated with GsTMx4 (black arrow, trace) for 5 min and DxS added at $t = 0$ min (black). (B) As in (A) but monitoring $[Na^+]_i$. 

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3.6 Discussion

3.6.1 Summary

In this chapter, I have been able to confirm using indo-1 Ca\textsuperscript{2+} imaging with FACS that there is indeed a Ca\textsuperscript{2+} rise in DP thymocytes after the addition of 1 µg/mL DxS. This rise has most of the hallmarks of that described by Tellam & Parish and was not caused by SOCE via STIM/Orai channel activation. However, using simultaneous Na\textsuperscript{+} imaging I discovered that there is also a concomitant large [Na\textsuperscript{+}]\textsubscript{i} rise, which notably, is independent of [Ca\textsuperscript{2+}]\textsubscript{o}. Furthermore, I found that Mg\textsuperscript{2+} blocked both rises in a concentration-dependent manner such that it was fully blocked with 30 mM [Mg\textsuperscript{2+}]\textsubscript{o}. I also observed that if background Ca\textsuperscript{2+} in these thymocytes was elevated by about 60 nM, both the Na\textsuperscript{+} and Ca\textsuperscript{2+} rises were blocked. Notably, I found the DxS rises were sensitive to lowering the temperature <30°C. These data suggest that the rises may be due to the activation of a non-selective cation-channel.

I also evaluated if blocking signalling elements abolished the rises seen. I have shown that blocking Src kinase abolished the DxS induced rises. Also, I show that blocking PLC and PI\textsubscript{3}K activation significantly inhibited both rises. Furthermore, the rises were decidedly diminished in mice deficient in either CD8β or LFA-1. In addition, block of cytoskeletal re-arrangement prevented the rises as did GsMTx4, suggestive of an involvement of a mechanosensitive element. Blocking PKC and PKA reduced both rises indicative of channel modulation by both kinases, consistent with TRP channels being the target.

3.6.2 Simultaneous Ca\textsuperscript{2+} and Na\textsuperscript{+} imaging using FACS

Ion imaging with FACS has advantages and at least one drawback. The advantages are that it is simple to implement and provides data from a very large number of cells (at least 10\textsuperscript{6} cells over 15 min). Due to the manual handling of the
container, there is a gap in acquisition of about 10–40 s after the addition of DxS. If a rise and decay had occurred during this time, it would have escaped detection. Single-cell imaging might be the technique to circumvent this limitation.

Imaging of \([\text{Ca}^{2+}]_i\) in thymocytes has been done since the early days of \(\text{Ca}^{2+}\) imaging (Davey \textit{et al.} 1998, Hesketh \textit{et al.} 1983, Mahaut-Smith & Mason 1991, Tsien \textit{et al.} 1982). Using ionomycin, I was able to show that the rise observed was well within the linear range of indo-1 and far from dye saturation.

Novel in this chapter is the extension of FACS imaging to include the simultaneous tracking of \([\text{Na}^+]\). When using ANG-2, there is moderate spill from the free indo-1 emission into the \(\text{Na}^+\) fluorescence that required compensation. In contrast to \(\text{Ca}^{2+}\) imaging and estimating the relevant concentrations, I was only partially successful in precisely quantifying the change in \([\text{Na}^+]\). The reasons are that thymocytes responded poorly to AmpB. I suspect that exposure to AmpB would have caused a prolonged and considerable depolarisation to close to 0 mV with all its consequences that likely lead to apoptosis. Chelation of \([\text{Ca}^{2+}]_o\), using EGTA, may reduce this issue. However, despite this, I am quite confident that the estimate for \([\text{Na}^+]\) is in the ballpark, as when bracketed against the rise caused by ConA (Segel \textit{et al.} 1979), largely the same value was obtained.

3.6.3 Characteristics of the rises

In agreement with Tellam and Parish (1987), I found the \([\text{Ca}^{2+}]_i\) rise resulted from influx across the PM as it was abolished when \([\text{Ca}^{2+}]_o\) was nominally <50 nM (1 mM EGTA, as estimated by MaxChelator). Under these conditions, the \(\text{Na}^+\) influx remained unchanged.

In contrast to the time course in Tellam and Parish (1987), I did not observe a biphasic rise. Specifically, ~1 min following the addition of DxS, a small rise was
detected when monitoring $[\text{Ca}^{2+}]$ with a spectrometer. However, this rise was not apparent in my data. This discrepancy might be explained by the condition under which the data was acquired.

Firstly, in flow cytometry experiments, the cells are in suspension when imaged. In contrast, during spectrometry, cell adhesion to the glass cuvette is possible and, perhaps as observed in T cells, adhesion to the glass may have primed the cells. Stimulation of adhesion–primed T cells is associated with larger $\text{Ca}^{2+}$ rises, thought to be a consequence of increased store filling (Randriamampita et al. 2003). Secondly, distinct from flow cytometry which detects fluorescence emission from cells as they rapidly pass by the detector (~$800$/s), the signal detected during spectrometry recording is acquired from the total cell sample and corresponds to a spatial mean.

I could not detect any difference in time courses between either the $\text{Ca}^{2+}$ or $\text{Na}^{+}$ rise as the values for half-activation were indistinguishable (~11 min). However, this does not exclude the possibility that one of the rises may have preceded the other as the variability in time course may have prevented its detection. In fact, in the next Chapter, I observe that when recorded at 30°C, the rise in $\text{Na}^{+}$ precedes that of $\text{Ca}^{2+}$.

Both rises also reached a plateau, with the rate of rise marginally faster for that of $\text{Na}^{+}$ than $\text{Ca}^{2+}$. This difference is likely due to the sensitivity and property of the dye rather than the underlying influx as both time courses when superimposed were indistinguishable (see Figure 3.4A).

3.6.3.1 $\text{Ca}^{2+}$ rise

There is little disagreement about the background concentration of $\text{Ca}^{2+}$ in thymocytes with values between 90–120 nM reported. For my estimation, I used
a value of 110 nM and calculated that the change in [Ca^{2+}]_i was 165 ± 11 nM, consistent with earlier reports (Tellam & Parish 1987, Weston et al. 1991).

3.6.3.2 Na\(^+\) rise

I found that [Na\(^+\)]_i rose by ~20 mM as determined by the fluorescence change from ANG-2. This value was bracketed against the known rise caused by ConA (Segel et al. 1979). I note that in contrast to Ca\(^{2+}\), Na\(^+\) is not buffered intracellularly, but some of it can be sequestered into endosomal compartments (Nass et al. 1997, Xiang et al. 2007). I am unable to estimate how much Na\(^+\) enters the cell but given this large [Na\(^+\)]_i change, a considerable depolarisation must be associated with it. Resulting from the depolarisation and the concomitant [Ca^{2+}]_i rise, activation of known Kv1.3 and Kc3.1 would provide the “counter currents” to the Na\(^+\) influx. These counter currents act to hyperpolarise membrane, with the aim of restoring the resting V_{m}. This likely explains why these cells, despite being electrically “silent”, express such K\(^+\) channels at a considerable density (Lewis & Cahalan 1988, Mahaut-Smith & Mason 1991). It is expected that some of the counter currents may also stem from homeostatically active transporters like the NCX (see next), Na/H exchanger and Na/K-ATPase. The observation of a large conductance change is consistent with my observation made during my Honours project (Feakes 2012) that when cells were patched and exposed to Dxs, there was a large inward current after ~5 min which ultimately exceeded the range of the voltage-clamp amplifier.

These considerations imply that the permeation of Na\(^+\) is very likely much larger than that of Ca\(^{2+}\) by several orders of magnitude. It requires the consideration of a channel not only with large partial permeability for Na\(^+\) but also of large conductance.
3.6.3.3 Involvement of NCX

I found that the amplitude of the Ca$^{2+}$ rise was partially dependent upon the Na$^+$ entry from NCX transport in reverse mode, as it was reduced by ~40% when [Na$^+$]o was replaced with NMDG and when NCX was inhibited by 3 µM SN-6 or 0.5 µM YM244769. The incomplete block may have at least two explanations. Firstly, the rises are likely the result of permeation via a non-specific cation channel which may even be co-localised with NCX as reported for TRPC6 (Syyong et al. 2007). It is worth pointing out that the block of NCX did not affect the [Na$^+$]i, consistent with the statement above that Na$^+$ imaging tracks much higher concentrations than those associated with Ca$^{2+}$ as a consequence of NCX activity. Secondly, since I did not concomitantly block NCKX, the possibility exists that the remainder was due to exchange via NCKX. While I think that this is unlikely, I note that relatively high expression of NCKX4 mRNA was detected in rat thymic tissue (Li et al. 2002b).

3.6.3.4 Plateau phase

A plateau of [Ca$^{2+}$]i and [Na$^+$]i elevation is reached if the driving force for the current approaches the reversal potential or if the influx of Na$^+$ is matched by an outflow of ions (counter currents). Since the reversal potential for non-specific cation channels is around 0 mV, it is possible that $V_m$ may depolarise close to this value. Whilst I cannot exclude this consideration due to the inability to keep these cells in voltage-clamp, I think that the second possibility is more likely due to the fact that upon a large and slow depolarisation, homeostatic mechanisms would kick in quickly. Consequently, the plateau of Na$^+$ is likely the “driver” for that of Ca$^{2+}$. The small influx of Ca$^{2+}$ is then the result of limited expression of NCX/NCKX in the PM.
3.6.4 Lack of store involvement

The experiment in which the extracellular Ca\(^{2+}\) was lowered with EGTA does not rule out the possibility that a small transmembrane Ca\(^{2+}\) influx is amplified by intracellular release akin to what is observed for cardiac myocytes (Franzini-Armstrong et al. 1998). Two lines of evidence rule this possibility out. Firstly, my results confirm the observation made by Tellam and Parish (1987), in that intracellular release caused by ConA when added during the plateau phase remained additive and had a fast onset (Figure 3.2A). Furthermore, store depletion using SERCA inhibition in cells with a considerable ER leak together with Orai block, did not significantly reduce the DxS-induced cation influx either (Figure 3.10D). It is therefore highly unlikely that elements of SOCE are involved. Secondly, I used norgestimate to activate a large intracellular Ca\(^{2+}\) release and was able to dissect the contribution of IP\(_3\)R to the rise using XestC. The IP\(_3\)R-mediated component had an intermediate onset, much faster than the DxS rise. Even with IP\(_3\)R largely blocked, the rises remained ruling out the possibility of IP\(_3\)R-modulation of channels like TRPC6 (Patterson et al. 2004). Likewise, the idea that IP\(_3\)R in the PM could underpin the rises is very unlikely (Khan et al. 1992a, Khan et al. 1992b).

These results also show that thymocytes contain the elements for SOCE and ROCE, but neither of them seems to be employed for the DxS rises.

3.6.5 Mg\(^{2+}\) sensitivity

I found that the cation rise was blocked Mg\(^{2+}\) in a concentration dependent way at >10 mM, with a full block around 25 mM. The target(s) of this block remain unclear but could involve a TRP channel and perhaps elements in the signalling cascade.
In the first case, the mechanism of block at a high divalent concentration may likely be unspecific and not physiologically relevant (Hille 2001). Channel activation may shift to more depolarised potentials (Kostyuk et al. 1982) caused by gating modification by the surface charge effect (Frankenhaeuser & Hodgkin 1957) or actual binding or competitive unbinding in the channel pore (Armstrong & Bezanilla 1973, Obukhov & Nowycky 2005). The latter mechanism is likely in the inhibition of TRPV3 by \( [\text{Mg}^{2+}]_o \). Specifically, Luo et al. (2012) show that inhibition of TRPV3 in the presence of 10 mM \( [\text{Mg}^{2+}]_o \) was dependent upon \( \text{Mg}^{2+} \) interaction with an aspartic acid residue in the outer pore region.

Alternatively, at high concentrations, \( \text{Mg}^{2+} \) may cross the membrane via TRPM6 or TRPM7 and cause a block from the inside as described for TRPV3, -C5, -M1, -M3, -M6 and -M7 channels (reviewed by Bouron et al. 2015, Luo et al. 2012, Obukhov & Nowycky 2005, Rampino & Nawy 2011). In this second case, \( \text{Mg}^{2+} \) would have to pass across the membrane for example as indicated above and affect the signalling cascade as a cofactor. ATP binding to kinases and some serine/threonine phosphatases (PP2C) are known to be \( \text{Mg}^{2+} \)-dependent (Kanellopoulou et al. 2019, Wera & Hemmings 1995). Given that increased \( [\text{Mg}^{2+}]_i \) generally leads to improved enzymatic activity, elements of the signalling cascade may be modulated.

### 3.6.6 Elevated background \([\text{Ca}^{2+}]_i\): associated with block

I have been able to show that if background \([\text{Ca}^{2+}]_i\) was elevated by \(~60\) nM (see Figure 3.2B—D), the addition of DxS did not cause any further rise. Several drugs used during this project (e.g. ConA, Pyr3, FFA, CPZ and NS8593) “blocked” the rises, due to this mechanism. In some cases, it was possible to lower the respective concentration to still allow for either partial or full rises (shown in Figure 4.10A & B and later in Figure 4.14A).
The mechanism behind this block remains unclear. Potential explanations could include, negative regulation of the protein tyrosine phosphatase CD45 (a crucial regulator of Lck activation; Ostergaard & Trowbridge 1991, Wang et al. 2000) or activation of the serine/threonine phosphatase calcineurin (phosphatase 2A; Zheng et al. 2019). In this instance, the modulated phosphatase activity would “counter” the kinase activity required for the signalling pathway(s) discussed below. The fact that with store emptying (Figure 3.10E), “blocking” concentrations of intracellular Ca\(^{2+}\) were apparent at the time of DxS addition, yet the rises could still be evoked, may point to spatial and temporal Ca\(^{2+}\) modulated signalling requirements downstream of Lck activation, perhaps even Ca\(^{2+}\)–CAM modulation of channel gating. Notably, most TRP channels are modulated by [Ca\(^{2+}\)].

In addition to the [Ca\(^{2+}\)]\(_{i}\) elevation, other limiting factors could be involved. A common proximal signalling step might not only lead to the Ca\(^{2+}\) rise by 60 nM but may also cause a concurrent change in lipid composition (such as PIP\(_{2}\) depletion) blocking channel gating (Borbiro et al. 2015, Tsuchiya et al. 2018).

### 3.6.7 Temperature sensitivity

Unexpectedly, I found that lowering the temperature to <30°C abolished both rises (see Figure 3.2F and Figure 3.4D). The abolition of both rises may be caused by an elevation in background [Ca\(^{2+}\)]\(_{i}\) dependent upon the thermally sensitive STIM1 molecule (Xiao et al. 2011). This perhaps involves temperature dependent conformational change of the STIM1, its clustering in ER-PM junctions and consequent activation of Orai1 channels independent of store Ca\(^{2+}\) release (Nwokonko et al. 2019, Xiao et al. 2011). Notably in preselection DP thymocytes, the expression of Orai1 is low, however a 3-fold increase in its expression has been reported in DP that have commenced positive selection (Appendix 6.6). In Jurkat cells, heat (41°C) induced STIM1 activation was shown by Xiao et al. (2011)
to enable progressive gating of Orai1 channels notably as the applied heat was reduced back to 25°C. The onset of the Ca\textsuperscript{2+} influx occurred at ~ 37°C. This store independent STIM1/Orai1 activation mechanism and subsequent sustained Ca\textsuperscript{2+} entry may also provide a mechanism for negative regulation of the non-selective cation entry channel. Such an increase in background Ca\textsuperscript{2+} might explain the loss of the DxS induced cation influx when the temperature is <30°C.

Furthermore, it is possible that during the fluorescent dye loading procedure, the incubation at 37°C for 1 h may have been sufficient to thermally activate a conformational change of STIM1 and puncta formation at the PM that is independent of store Ca\textsuperscript{2+} depletion. Subsequent lowering of the temperature could perhaps then result in Orai1 channel activation and a sustained rise in basal [Ca\textsuperscript{2+}]\textsubscript{i} in mature thymocytes. However, based on the findings presented by (Xiao et al. 2011) it is hypothesised that this Ca\textsuperscript{2+} rise would decay over time when thymocytes are maintained at RT as depicted in Figure 3.2F (inset).

It is noted that in thymocytes kept on ice, background [Ca\textsuperscript{2+}]\textsubscript{i} is indeed elevated; reminiscent of a similar temperature-sensitivity found in platelets (Oliver et al. 1999). This rise cannot be explained by a change in indo-1 fluorescence intensity. The fluorescence intensity of many fluorescent probes increases at lower temperatures. However, as the bound and free states of indo-1 respond differently, the R indo-1 (F\textsubscript{b}/F\textsubscript{f}) reportedly reduces as the temperature is decreased (Oliver et al. 2000).

While the mechanism of thermal sensitivity of the DxS induced cation influx may indicate that there is a critical step in the signalling leading up to gating that is highly temperature-sensitive it may also point to a temperature-sensitive non-specific cation channel like TRPV1–4, TRPM3 or TRPA1 (see next chapter).
3.6.8 **Mechanosensitivity**

I have been able to fully block both rises using 3 µM GsMTx4. As this tarantula toxin is inserted into the lipid bilayers and alters lipid packing, it is postulated that it may prevent a mechanosensitive element from being exposed to membrane tension stress (Gnanasambandam et al. 2017, reviewed by Gottlieb et al. 2007, Suchyna et al. 2004). This block suggests an involvement of a mechanosensitive element, the molecular nature of which remains unclear. However, candidates include Piezo1 and several TRP channels (TRPV1, TRPV2, TRPV4, TRPC1, TRPC6 TRPM2 TRPM4 and TRPM7). The activation and inactivation properties of Piezo1 described earlier (1.7.2, p. 82) would suggest that this channel, if involved, is upstream of activation of a second non-selective cation channel.

![Figure 3.18 Proposed mechanosensitive involvement](image)

**Figure 3.18 Proposed mechanosensitive involvement**

Without TCR engagement Dxs binding to HS binding sites on CD8β results in both (1) transmission of mechanical load directly to the CD3 tails and to the local PM which promotes both gating of local Piezo1 channels and (2) both activation of Lck. A transient local Ca²⁺ influx within the region of the CD3 membrane bound tails enhances CD3 conformational change exposing Lck phosphorylation sites. (3) activation of an inside out signal and cytoskeleton reorganisation leads to LFA-1 conformational change (4) Synergistic outside in signalling enable formation of the LAT signalosome and PLC-γ1 activation.
Whether this toxin has alternate inhibitory targets such as mechanosensitive G-proteins, such as the highly expressed angiotensin II type 1 receptor (Storch et al. 2012), is unknown. How the addition of GsMTx4 might alter activation of mechanosensitive transduction proteins, apart from ion channels, in thymocytes remains to be elucidated.

3.6.9 **Signalling steps upstream of the rises**

Results presented in this chapter indicate that the DxS [Ca$^{2+}$]$_i$ rise occurs downstream of signalling pathways likely triggered by DxS cross-linking of CD8β and LFA-1 as both rises were significantly reduced in thymocytes from both CD8$^{β−/−}$ and LFA-1$^{−/−}$ mice. This step is likely the most proximal in the steps required to gate the channel. In addition, in peripheral CD8$^+$ lymphocytes that normally do not respond to DxS, when sialylation was cleaved, using neuraminidase, the rises were re-established (Figure 3.12D). This indicates that this post-translational modification is a critical molecular regulator and it impedes the signalling cascade leading to the activation of the non-specific cation channel(s).

Since DxS does not stimulate the TCR directly but elements of the coreceptor complex, the signalling that leads to the rises, may likely involve synergistic amplification of concurrent signalling pathways. This idea is consistent with the observation that in thymocytes from these KO animals, both rises were significantly diminished (Figure 3.12A-B & E-F).

An additional pathway that might augment local signalling is likely dependent upon Lck tyrosine phosphorylation (Src kinase) as it was potently blocked by 0.1 μM PP2, a pan Src-family kinase blocker that potently blocks Lck and Fyn (Hanke et al. 1996).
The finding that both fluxes were blocked in conditions where resting Ca\(^{2+}\) was stably elevated by \(\sim 60\) nM, prior to the addition of DxS, may suggest altered phosphatase and/or kinase activity. Signal transduction can be modulated by PKC or PKA phosphorylation of effector molecules. In addition, PKC and PKA phosphorylation of some ion channels, including some TRP channels, can enhance their open probability. Using Gö9683 or H-89 to broadly inhibit PKC or PKA, respectively, I found the DxS rises were only reduced by \(\sim 35\%\). This finding suggests PKC and PKA activation likely modulates the signalling mechanism, in contrast to the critical role of Src kinase(s) in enabling it.

The rise was largely blocked when PLC, most likely the γ1 isoform, was blocked with 1 µM edelfosine. At this concentration, this drug is not toxic to thymocytes and does not stimulate a mitogenic response (Cardile et al. 1997). In addition, it is below the concentration used to inhibit PI3K in mouse fibroblasts (IC\(_{50}\) = 35 µM; Berggren et al. 1993). Furthermore, unlike U73122, a structurally different PLC inhibitor, edelfosine does not have the side effect of partial PLC activation if left in the suspension (Thyagarajan et al. 2009). However, given that in mouse fibroblasts, the reported the IC\(_{50}\) of edelfosine for PLC inhibition was 9.6 µM (Powis et al. 1992), the much smaller concentration used here may have resulted in an incomplete block with small rises remaining. However, concentrations >1.5 µM have been shown to decrease thymocyte viability (Cardile et al. 1997) and massively increased [Ca\(^{2+}\)] in thymocytes (Figure 3.10A inset, red). This finding therefore is consistent with a view that PLC-γ is downstream of CD8β, LFA-1, Lck and PI3K, the knock-out or block of which also fully abolishes the cation rise.

Since PIP\(_2\) hydrolysis by PLC-γ1 results in the production of DAG, channel activation may be caused by either PIP\(_2\) depletion or DAG increase. Examples of such channels for the former are TRPM1, -V3 and -V4 and for the latter some members of the TRPC subgroup, TRPA1 and -V2. However, I have been able to
rule out that the other signalling molecule, namely IP₃, is not involved in the cation rise since the block of IP₃R by XestC did not show any effect even when accounting for its slow on-rate.

3.6.10 **Candidate channels activated downstream of Dxs**

Altogether, the results from this chapter, do not point to a specific channel underlying the Dxs rises. Rather, many of the characteristics, such as enhanced channel activity following Src kinase phosphorylation and/or PIP₂ depletion, mechanosensitivity and coupling to NCX activity can be found spread across a number of non-selective channels from the TRPC, TRPV and TRPM subgroups and Piezo1. To help narrow the scope of the next step, a list of potential candidates was compiled and is presented below in Table 3.2. Included are channels which have properties that are modulated by mechanisms that affect the cation influx. TRP channels listed on the left side contain more of the positive (green shading) characteristics required for the Dxs induced cation rise and so were considered as the primary candidates for investigation. TRP channels listed toward the right of the table have fewer positive characteristics, however they cannot be discounted at this point. Other members of these subfamilies that do not share at least four of the properties listed were omitted from Table 3.2. It needs to be pointed out that some of these properties were obtained in heterologous expression systems and therefore should be considered with caution.
### Table 3.2 Candidate channels

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<th>Characteristics</th>
<th>TRPV1</th>
<th>TRPV3</th>
<th>TRPV4</th>
<th>TRPC6</th>
<th>TRPA1</th>
<th>TRPV2</th>
<th>TRPM1</th>
<th>TRPC2</th>
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<td>Src kinase modulation</td>
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<td>STIM1 interaction</td>
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<td>IP3-R interaction</td>
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<td>Coupled to NCX</td>
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<td>PLC-γ1 dependent</td>
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<td>PIP2 binding</td>
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<td>Activated by DAG</td>
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<td>Activated by AA</td>
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<td>PKC modulation</td>
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<td>Mechano sensitivity</td>
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<td>Thermal sensitivity</td>
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<td>Ca2+ sensitivity</td>
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<td>Mg2+ sensitivity</td>
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</tr>
</tbody>
</table>

- Positive evidence. ♩ Spike in DP population (ImmGen database). ♩Contrary evidence: dependent on cell type and expression system. □ Indirect activation. ▲ Rise enables activation. ▼ Depletion enables activation. ↑ Potentiation and ↓ inhibition. References can be found at the end the reference section.

References:

- [1–9]
- [10–12]
- [13–21]
- [22–30]
- [31–39]
- [40–44]
- [45]
- [46–48]
- [49–54]
- [55–57]
- [58–60]
- [61–64]
- [65–66]
- [67–71]
- [72–73]
I have considered the exquisitely mechanosensitive Piezo1 in this selection of channels for the following reason. Recently reported evidence shows that a discrete transient Ca\(^{2+}\) influx enabled by Piezo1 activation facilitates the TCR/CD3 signal transduction in mature T lymphocytes (Liu et al. 2018). While DxS is not thought to stimulate the \(\alpha\beta\)TCR, its binding to the CD8\(\beta\) coreceptor is perhaps sufficient to initiate a mechano-sensitive (i.e. Piezo1) and biochemical (i.e. Lck activation) signal transduction. However, the rapid, transient activation and sustained inactivation properties of Piezo1 do not match with the delayed onset of the Ca\(^{2+}\) and Na\(^+\) rises. For this reason, Piezo1 would likely require activation of a second channel to account for the plateau phase of both rises.

In the following chapter, I will present results that focus on the non-specific cation channels listed in Table 3.2, with the aim of identifying if any of these channels could underpin the DxS rises. Notably, as shown in Table 3.2, TRPV1, TRPC6, TRPA1, TRPC2 and TRPC7 can be directly activated by DAG. Having this characteristic will provide the starting point for channel investigation.
4 Evaluation of candidate channels

4.1 Introduction

Identification of ion channels and transporters that have functional roles in enabling discrete and global changes in \([\text{Ca}^{2+}]_i\) during DP thymocyte selection and differentiation is far from complete. While it appears SOCE channels are activated in response to highly avid TCR–pMHC engagement during thymocyte–stromal cell interaction, this mechanism of \(\text{Ca}^{2+}\) entry is not required for signalling leading to positive selection. Furthermore, channels that might enable synergistic \(\text{Ca}^{2+}\) entry and so modify signal transduction where TCR–pMHC engagement is suboptimal, while predicted, are yet to be identified.

In peripheral T lymphocytes, \(\text{Ca}^{2+}\) entry via TRP channels (e.g. TRPC3 and TRPV1) and Piezo1 have been shown to synergistically enhance TCR signal transduction (Bertin et al. 2014, Liu et al. 2018, Philipp et al. 2003, Wenning et al. 2011), while TRPA1 linked inhibition of TRPV1 activity reduces TCR induced \(\text{Ca}^{2+}\) entry in CD4 T-cells (Bertin et al. 2017). Hypothesised to mimic an endogenous heparin sulfate evoked signalling cascade, the addition of DxS results in a sustained \([\text{Ca}^{2+}]_i\) increase of \(\sim 170\) nM and a concomitant \([\text{Na}^+]_i\) increase of \(\sim 20\) mM. This \(\text{Ca}^{2+}\) and \(\text{Na}^+\) entry occurs in preselection DP thymocytes, and while dependent upon CD8β and LFA-1 co-stimulation, it is independent of αβTCR stimulation by pMHC-I engagement (Simon Davis 2015). Notably the cation influx appears to be positively modulated by warming to 37°C, membrane tension, AA, phosphorylation and the chemical 2-APB. Multimodal stimuli variably sensitise TRPV, TRPM, TRPA1 and TRPC channel gating, however, no single channel appears to be sensitised by all these gating modulators. Rather, the results of pharmacological testing presented in this chapter suggest that while
TRPV3 is the likely channel, the involvement of Piezo1 and TRPM1 remains to be clarified.

In recent years, evidence of Ca\(^{2+}\) permeable TRP channels and Piezo1 expression in T lymphocytes has emerged (reviewed by Bertin & Raz 2016, Khalil et al. 2018, Liu et al. 2018, Majhi et al. 2015). However, the interplay of signalling pathways and multiple gating mechanisms that lead to TRP channel activation, together with significant functional redundancy, heteromeric channel formation, functional differences between cell type, and a dearth of selective activating and inhibitory drugs, often means the functional role of many of these channels remains obscure. Furthermore, the patterning and duration of Ca\(^{2+}\) rises likely involve multiple mechanisms regulating \([Ca^{2+}]_i\) (reviewed by Christo et al. 2015, Feske 2007, Feske et al. 2015, Nohara et al. 2015, Robert et al. 2011, Winslow et al. 2003).

Considering these challenges, this chapter explores the potential involvement of a number of TRP and Piezo channels in contributing to the sustained \([Ca^{2+}]_i\) and \([Na^+]_i\) increases in DP thymocytes following the addition of DxS.

Results presented in the previous chapter indicate that following the addition of DxS, activation of one or more signalling pathways precedes activation of the cation influx. Initiation of the rises was strictly dependent upon CD8\(\beta\) and LFA-1, most likely as they are cross-linked by DxS. Subsequent Src kinase activation and tyrosine phosphorylation of adaptor molecules such as SLP-76 (Simon Davis 2015) facilitate recruitment and activation of PLC-\(\gamma\)1 and PI\(\beta\)K (Shim et al. 2011). Enzymatic activity of both PLC-\(\gamma\)1 and PI\(\beta\)K can locally alter the membrane lipid composition, in particular PIP\(_2\), a significant modulator of TRP and Piezo1 channel activity.
The hydrolysis of PIP₂ by PLC-γ1 increases the availability IP₃ and DAG. As presented in 3.6.2, IP₃ activation of IP₃R and ER store Ca²⁺ release do not appear to be a necessary component of the DxS rises. Rather channel activation is proposed to involve PIP₂, DAG or a downstream metabolite such as AA. As with PIP₂, DAG and AA have also been found to modulate channel gating of some TRPC, TRPA and TRPV members. The finding that the rise is potently blocked by prior addition of GsMTx4, suggests that a mechanosensitive element is likely involved. These characteristics provide the starting point for evaluating potential candidate channels.

4.2 Materials

Details of the solutions and chemicals commonly used in cell preparation and flow cytometry experiments can be found in Chapter 2. Table 4.1 provides details of the concentrations and different protocols of the various inhibitory or activating chemicals used to assess the role of channels in the DxS rises. In most instances, the chemical was added at \( t = -1 \) min followed by DxS at \( t = 0 \) min. When checking the effect of the chemical on its own, it was added at \( t = 0 \) min.
Table 4.1 List of chemicals

Time and details of when the relevant chemical was added to the cell suspension in flow cytometry experiments where DxS was also added at $t = 0$.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Putative Target</th>
<th>Action</th>
<th>Protocol</th>
<th>Conc. (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-APB</td>
<td>* TRPs</td>
<td>$t = -1$ min</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>A967079</td>
<td>TRPA1</td>
<td>inhibitor</td>
<td>$t = -5$ min</td>
<td>1</td>
</tr>
<tr>
<td>AMG9810</td>
<td>TRPV1</td>
<td>inhibitor</td>
<td>$t = -1$ min</td>
<td>0.5</td>
</tr>
<tr>
<td>Arachidonic acid</td>
<td>TRPV3, TRPM7</td>
<td>activator, inhibitor</td>
<td>$t = 0$ min</td>
<td>10</td>
</tr>
<tr>
<td>Capsazepine</td>
<td>TRPV1</td>
<td>inhibitor</td>
<td>$t = -1$ min</td>
<td>0.5</td>
</tr>
<tr>
<td>Gadolinium</td>
<td>TRPs</td>
<td>inhibitor</td>
<td>DxS not added</td>
<td>10</td>
</tr>
<tr>
<td>GSK2193874</td>
<td>TRPV4</td>
<td>inhibitor</td>
<td>$t = 1$ min</td>
<td>0.04</td>
</tr>
<tr>
<td>HC030031</td>
<td>TRPA1</td>
<td>inhibitor</td>
<td>$t = -1$ min</td>
<td>20</td>
</tr>
<tr>
<td>Hyperforin</td>
<td>TTRPC6</td>
<td>activator</td>
<td>$t = 0$ min</td>
<td>5</td>
</tr>
<tr>
<td>Na–ATP</td>
<td>TRPV3</td>
<td>activator</td>
<td>$t = 0$ min</td>
<td>100</td>
</tr>
<tr>
<td>NDGA</td>
<td>TRPM7</td>
<td>inhibitor</td>
<td>$t = -1$ min</td>
<td>4</td>
</tr>
<tr>
<td>Norgestimate</td>
<td>$\infty$TRPC6</td>
<td>inhibitor</td>
<td>$t = -1$ or $t = 1$ min</td>
<td>10–15</td>
</tr>
<tr>
<td>OAG</td>
<td>TRPC2,6,7 TRPA1, V1</td>
<td>activator</td>
<td>DxS not added</td>
<td>100</td>
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<td>SKF96365</td>
<td>#TRPC6</td>
<td>inhibitor</td>
<td>$t = -1$ or $t = 12$ min</td>
<td>5–15</td>
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<tr>
<td>NS8593</td>
<td>TRPM7</td>
<td>inhibitor</td>
<td>$t = -5$ or $t = 14$ min</td>
<td>10–15</td>
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<td>Ruthenium red</td>
<td>* TRPVs</td>
<td>$t = -1$ or $t = 12$ min</td>
<td>2.5 or 10</td>
<td></td>
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<tr>
<td>Tranilast</td>
<td>TRPV2</td>
<td>inhibitor</td>
<td>$t = -1$ min</td>
<td>75</td>
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</table>

* See Table 1.3 Pharmacological effects of 2-APB and ruthenium red; # pan TRPC channel inhibitor and Orai channel inhibitor; $\infty$ TRPC3, 6 antagonist and progesterone receptor agonist
4.3 Results

4.3.1 *In silico* data base screening of mRNA expression

To investigate the likely expression of channels in DP thymocytes considered as potential candidates, searches of genes in the Immunological Genome Project database (ImmGen; [http://www.immgen.org/databrowser/index.html](http://www.immgen.org/databrowser/index.html)) were undertaken for probe sets for mice. The data available were derived by profiling gene expression on Affymetrix microarrays in cells from 5-week-old C57/Bl6J mice. The DP thymocyte populations of interest are described in the αβ T cell data group (i.e. cells expressing the α and β chains of the TCR).

Adapted from the ImmGen site, Figure 4.1 shows the mRNA expression for genes coding for non-selective cation channels that reportedly have mechanosensitive properties: *Trpc1, Trpc6, Trpv1, Trpv2, Trpv4, Trpa1, Trpm3, Trpm7 and Piezo1 (Fam38a) and Piezo 2 (Fam38b)* (Dietrich et al. 2007, Gottlieb et al. 2008, Grimm et al. 2003, Haselwandter & MacKinnon 2018, Maroto et al. 2005, Rocio Servin-Vences et al. 2017, Spassova et al. 2006). These panels indicate high expression of *Trpv2, Trpm7 and Fam38a* (top row) across the αβT cell group and specifically in the different DP populations bounded by the red and black dashed lines. The remaining genes, in contrast, appear to be minimally expressed. High expression refers to the arbitrary numbers of transcripts given along the abscissa, where typically >120 indicates true expression (more than 95% probability), while <47 suggests the gene expression is unlikely or silent (Ericson et al. 2019). In addition, I have included the expression profiles of two indirectly mechanosensitive channels *Trpc7* and *Trpm4* (Gonzales et al. 2014, Liu & Montell 2015). The expression of *Tprc7* is also low, and notably Inada et al. (2006) did not detect *Trpc7* in thymocytes (Figure 1.13). Importantly, high mRNA expression of a gene in DP thymocytes does not necessarily indicate a similarly high level of protein expression in these cells (Schwanhausser et al. 2011, Tian et al. 2004).
Figure 4.1 Gene expression of proposed mechanosensitive channels

Relative mRNA expression of *Trpv1*, *Trpv2*, *Trpv4*, *Trpc1*, *Trpc6*, *Trpa1*, *Trpm3*, *Trpm4*, *Trpm7*, *Piezo1* and *Piezo2* transcripts in αβT cell populations (ImmGen database). The
expression is predicted by the arbitrary numbers of transcripts given along the abscissa, where typically >120 indicates true expression (more than 95% probability), while <47 suggests the gene expression is unlikely or silent (Ericson et al. 2019). In descending order, the four DP subpopulations framed by the dashed black lines are: all, blasts, small resting, and DP early positive selection 69+ thymocytes. The three DP subpopulations framed by the dashed red lines are DP 69− preselection, DP 69+ early positive selection, and DP early positive selection.

Considering the arbitrary values given in Figure 4.1, it appears that gene expression of Trpv1, Trpa1, Trpc6 and perhaps also Trpv4 in ‘preselection DP CD69− thymocytes’ may be silenced. Referring to Table 3.2, these four channels were predicted to be ‘good’ potential candidate channels. Therefore, I checked if the protein might nevertheless be present in the PM of DP thymocytes.

4.3.2 **Protein detection indicates mechanosensitive channels on the PM**

As the amount of gene expression does not necessarily indicate the presence of the protein in the PM, and, conversely, protein may be present despite negligible gene expression, ICC experiments were undertaken to check if channels thought to show mechanosensitive properties might be present. The cells were prepared following the protocol described in section 2.3.5, and the data was acquired on a BD LSRII flow cytometer. Commercially available extra-cellular Ab₁ against TRPC6, -C7, -V2, -V4 and -A1 (described in Table 2.4) were applied and detected by secondary staining with an Ab raised against the host species IgG and conjugated to the fluorochrome FITC (Ab₂). Owing to the lack of commercially available Ab₁ against an extracellular epitope on Piezo1, TRPV1, -M3, or -M7 the expression of these channels was not investigated.

The contour plots of CD8α against TRP channel expression are shown in Figure 4.2. From left to right, the four plots show the relative density and frequency of i) unstained cells, ii) anti-CD8α–AF647 plus Ab₂ (without Ab₁) stained cells, iii) anti-CD8α–AF647 plus Ab₁ (anti-TRP) preincubated with antigen (blocking) peptide (BP) plus Ab₂ stained cells, and iv) anti-CD8α–AF647 plus Ab₁ (anti-TRP)
plus Ab\textsubscript{2} stained cells which detect binding of the primary anti-TRP channel antibody. The threshold for detection of Ab\textsubscript{1} binding in CD\textsubscript{8\textsuperscript{hi}} cells was set as illustrated in (iii) by positioning the vertex of a quadrant gate (red) immediately to the right and beneath the main population density. The same quadrant gating was also copied into plots i, ii and iv. The relative frequency in these sample plots is indicated by the percentage value in each quadrant.

Examining the plots in which Ab\textsubscript{1} cell staining was included (iv), there were significant shifts of the respective population densities into the top right quadrant in all cases ($p_{KS} < 10^{-30}$). This indicates that several target channel proteins are likely present in the PM surface in CD\textsubscript{8\textsuperscript{hi}} thymocytes. Notably, the anti-TRPV4 Ab\textsubscript{1} binding was not specific to its immunogen. This is shown by the increased fluorescence detected in the TRPV4 Ab\textsubscript{1}–BP control (bottom row; iii) compared to the anti-CD8–Ab\textsubscript{2} only control (ii).

I note that immunocytochemical detection of TRP channel protein doesn’t imply that the channels are in a conductive state, however, their possible presence needs to be considered when interpreting subsequent data.

As indicated in Table 3.2, TRPC6 has been described as mechanosensitive channel that is positively modulated by Src kinase, directly activated by DAG, inhibited by GsMTx4 and may couple to NCX. These properties combined with the presumed expression of TRPC6 on the PM led to this channel being considered as the conduit to the cation rise.
Figure 4.2 Detection of mechanosensitive channels on the PM

Typical contour plots representative of ICC experiments investigating the possible presence of TRPC6, TRPC7, TRPA1, TRPV2 and TRPV4 channel protein on the surface of CD8<sup>hi</sup> thymocytes. The fluorescence intensities of the five Ab against TRP channels (rows) are shown in (iv) and the respective controls in columns (i–iii). Red lines indicate the quadrant gates set on the control Ab intensities in (iii) and copied (black) into the others. The relative percentages are given in each quadrant and the total number of cells top right outside the box.
4.3.3 OAG activated TRP channels are in the PM

Based on my ICC results, several DAG sensitive TRP channels may be present in the PM. These include TRPC2, -C3, -C6, -C7, -A1 and -V1. To check if some of these channels could be activated independently of DxS, I used the DAG analogue OAG (1-oleoyl-2-acetyl-sn-glycerol). As shown in Figure 4.3A, the addition of 50 µM OAG induced a transient rise in both [Ca\(^{2+}\)] and [Na\(^{+}\)]. (black and grey, respectively). Notably, the onset and rate rise were faster than that caused by DxS and, compared to the DxS control, these rises had a smaller amplitude for both Ca\(^{2+}\) (23 ± 6%, n = 6) and Na\(^{+}\) (38 ± 2%, n = 3). In addition, the Na\(^{+}\) rise was delayed and dissociated from the time course of Ca\(^{2+}\). This dissociation suggests that the addition of OAG may have activated more than one channel/transporter, either directly, or by changing membrane tension (Spassova et al. 2006), or may be the result of NCX activity.

Since many TRP channels are blocked by Gd\(^{3+}\), I checked if it blocked the OAG induced rises. For this purpose, I added 10 µM Gd\(^{3+}\) immediately prior to OAG at \(t = 0\) min. This concentration should potently inhibit TRPC6, TRPC3 and TRPA1, but not TRPC7 nor TRPV1 (summarised below in Table 4.2).

<table>
<thead>
<tr>
<th>Gd(^{3+}) (µM)</th>
<th>Inhibits</th>
<th>Activates</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>TRPA1 (Nagata et al. 2005)</td>
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<tr>
<td>10</td>
<td>TRPC6, C3 (Chen et al. 2017b, Inoue et al. 2001)</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>TRPC7 (Okada et al. 1999)</td>
<td>TRPV1, TRPC4, C5 (Plant &amp; Schaefer 2005, Tousova et al. 2005)</td>
</tr>
<tr>
<td>300</td>
<td>TRPV1 (Tousova et al. 2005)</td>
<td>2005, Tousova et al. 2005</td>
</tr>
</tbody>
</table>

Table 4.2 Modulation of TRP channels by Gd\(^{3+}\)
Shown in Figure 4.3, the addition of Gd\(^{3+}\) only partially inhibited the OAG evoked [Ca\(^{2+}\)]\(_i\) (B, ochre; \(n = 3, p_t < 0.001\)) and [Na\(^+\)]\(_i\) rises (C, ochre; \(n = 3, p_t < 0.01\)). This data suggests that OAG activates rises that are partially sensitive to Gd\(^{3+}\), consistent with TRP channels.

![Figure 4.3 OAG activates a Ca\(^{2+}\) and Na\(^+\) rise](image)

(A) Appended time courses for [Ca\(^{2+}\)]\(_i\) (black) and [Na\(^+\)]\(_i\) (grey) when OAG added at \(t = 0\) min. (B) Time course plots of the [Ca\(^{2+}\)]\(_i\) response when at \(t = 0\) min, either OAG (black); OAG plus Gd\(^{3+}\) (ochre) was added, or OAG plus 80 µM 2-APB was added (pink). (C) shows the time course plots for [Na\(^+\)]\(_i\) time in these same experiments.

I also checked the effect of 80 µM 2-APB on the OAG rise. 2-APB should inhibit TRPC channels and potentiate TRPA1 and TRPV1 (see Table 1.3, p. 108). The addition of 80 µM 2-APB also reduced the OAG evoked rises (Figure 4.3B & C, pink) indicating the presence of at least one TRPC channel. Together the Gd\(^{3+}\) and 2-APB data indicate that multiple TRP channels are likely expressed in the PM. Their presence may well confound some of the results presented later in this chapter.
4.3.4 Hyperforin activates a cation rise

I next checked if a non-selective cation rise could be elicited by 5 µM hyperforin, a proposed TRPC6 activator (Leuner et al. 2007). Shown in Figure 4.4A, the addition of hyperforin at \( t = 0 \) min (red arrow) caused a \([\text{Ca}^{2+}]_i\) rise that was notably faster and 39.6 ± 2.9\% smaller than the DxS \([\text{Ca}^{2+}]_i\) rise (red; \( n = 10, p_t <0.0001 \)). Additionally, a small concomitant \([\text{Na}^+]_i\) rise that was 16.8 ± 0.6\% of the DxS peak amplitude was also observed (Figure 4.4B; red, \( n = 3, p_t <0.001 \)). This data is consistent with the idea that TRPC6 channels in the PM can be activated.

![Figure 4.4 Hyperforin evokes a cation influx](image)

(A) Time course of \([\text{Ca}^{2+}]_i\) when hyperforin was added at \( t = 0 \) min (red arrow/trace) compared with the control DxS rise (blue). (B). As in (A) but monitoring \([\text{Na}^+]_i\).

4.3.4.1 TRPC6 inhibition does not abolish DxS cation rise

To determine if TRPC6 played a role in the DxS \([\text{Ca}^{2+}]_i\) rise I checked if the DxS cation rise could be inhibited either by norgestimate or SKF96365 (1-(\(\beta\)[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl)-1H-imidazolehydrochloride), both non-selective blockers of TRPC6. In mouse smooth muscle cells, 10 µM norgestimate (IC\(_{50}\) ~5 µM; Miehe et al. 2012) and 5 µM SKF96365 (IC\(_{50}\) = 4.25 µM; Inoue et al. 2009, Inoue et al. 2001) have been shown to markedly inhibit TRPC6...
channel activation. Notably both these drugs act on other targets. In Figure 3.10F (grey), I have shown that 15 µM norgestimate on its own caused a large and sustained [Ca^{2+}]_i rise in these cells, indicative of SOCE. With such a rise, the DxS rise is not apparent and likely inhibited.

To circumvent this problem, I investigated the effect of lowering the hyperforin concentration. Shown in Figure 4.5A, the addition of 12 µM norgestimate on its own caused a small [Ca^{2+}]_i rise (grey). When added at t = 7 min after DxS (aqua), the [Ca^{2+}]_i rise was not inhibited (n = 3, p_t = 0.92). Reducing the norgestimate concentration further, to <10 µM, I found that resting [Ca^{2+}]_i (n =6, p_t = 0.52) remained stable. Notably, when added 1 min prior to DxS, <10 µM norgestimate did not significantly block the DxS induced [Ca^{2+}]_i rise (Figure 4.5B). This data indicates that a putative block of TRPC6 does not reduce the Ca^{2+} rise. To eliminate the notion that 12 µM norgestimate might not have been sufficient to block TRPC6 and the confounding issue of background Ca^{2+} elevation, I checked if SKF96365 could block the rise.

Similarly, the addition of 10 µM SKF96365 did neither inhibit the [Ca^{2+}]_i nor the [Na^+]_i rise (Figure 4.5C & D, black; n = 3, p_t = 0.84; p_t = 0.82, respectively). Significantly, higher concentrations of SKF96365 have been shown to inhibit the SERCA pump in thymocytes (Mason et al. 1993). This is apparent in Figure 4.5E & F (black traces), where, following preincubation with 3 µM YM58483 to block SOCE and DxS addition at t = 0 min, 20 µM SKF96365 added at 12 min (black arrow) caused a transient [Ca^{2+}]_i rise but did not markedly affect the [Na^+]_i rise suggestive of a tonic Ca^{2+} leak from either stores and/or mitochondria.
Figure 4.5 TRPC6 channels can be activated with hyperforin

(A) Time courses of [Ca$^{2+}$]: when 12 µM norgestimate only was added at $t = 0$ min (grey) and when added at $t = 12$ min (aqua) (B) Normalised peak DxS [Ca$^{2+}$]: amplitudes following 5 min preincubation with 3, 4, 5, 6, 7, 8, 9 and 12 µM norgestimate (dashed line indicates control peak amplitude). (C) Time courses of [Ca$^{2+}$]: when 10 µM SKF96365 was added at $t = 0$ min (red arrow/trace), the control DxS rise (blue) and when SKF96365 (black arrow/trace) was added prior to adding DxS at $t = 0$ min. (D) As in (C) but monitoring [Na$^+$]. (E) Time course of [Ca$^{2+}$]: when preincubated with 3 µM YM58463 (orange arrow), before adding DxS at $t = 0$ min (grey arrowhead) and 20 µM SKF96365 at $t = 12$ min (black). (F) As in (E) but for [Na$^+$].
Both data sets are not consistent with TRPC6 involvement in the DxS rise. However, it is noted that the analysis of the results is confounded by the \([\text{Ca}^{2+}]_i\) rise from of intracellular stores. Therefore, to add more evidence that TRPC6 is not involved, I checked if 2-APB, another non-specific inhibitor of TRPC channels (Lievremont et al. 2005, Viitanen et al. 2013, Xu et al. 2005) could block the rises.

As detailed in Table 1.3, 2-APB has many targets. Concentrations of 30—300 µM progressively inhibit TRPC3, -C6 and -C7 channels and activate TRPV1, -V2 and -V3 channels (Chung et al. 2004, Hu et al. 2004, Lievremont et al. 2005). Having previously found that a stable rise in background \([\text{Ca}^{2+}]_i\) has an inhibitory effect on the DxS rise, I checked the effect of 30–100 µM 2-APB alone. Shown in Figure 4.6A (aqua), the addition of up to 100 µM 2-APB did not markedly elevate background \([\text{Ca}^{2+}]_i\). Consequently, I used 70 µM 2-APB (Figure 4.6B, aqua; \(n = 5, p_t = 0.62\)), a concentration below the block of PMCA (Peppiatt et al. 2003). In Figure 4.6C & D, the DxS rises are presented with the respective control (blue and green). In contrast to the expected block by 70 µM 2-APB, there was a large potentiation of both the \([\text{Ca}^{2+}]_i\) and \([\text{Na}^+]_i\) rises (black, 186 ± 29%; \(n = 4, p_t > 0.001\) and 145 ± 14%; \(n = 3, p_t > 0.05\), respectively). I found no difference between the two extents of potentiation (\(n = 5; p_t = 0.36\)) and the rate of rise of either cation (\(n = 5, p_t = 0.48\)). Consistent with the data presented above, these results do not suggest TRPC6 underlies the cation influxes.
Figure 4.6 2-APB potentiates both rises

(A) Time course of background \([\text{Ca}^{2+}]_i\) (light blue) and \([\text{Na}^+]_i\) (light green) in response to 30, 60, 75 and 100 µM 2-APB (arrows). (B) Time courses of \([\text{Ca}^{2+}]_i\) (light blue) and \([\text{Na}^+]_i\) (light green) after exposure to 70 µM 2-APB added at \(t = 0\) min. (C) Time course of DxS \([\text{Ca}^{2+}]_i\) control (blue) and when 70 µM 2-APB was added 1 min prior to DxS (black). (D) Same as in (C) but for \([\text{Na}^+]_i\).

However, because unspecific effects of 2-APB could have possibly confounded these results, additional experiments were undertaken using TRPC1,3,6,7\(^{-/-}\) mice, with the aim of obtaining the best evidence regarding TRPC6, and coincidently TRPC1, 3 and 7.

4.3.4.2 The DxS \([\text{Ca}^{2+}]_i\) rise occurs in TRPC1,3,6,7\(^{-/-}\) thymocytes

If TRPC6 channels were involved in the DxS rises, then the rises should be abolished in TRPC1,3,6,7\(^{-/-}\) mice. It is important to note that, in contrast to the commonly used C57BL/6J mice, in these experiments the TRPC1,3,6,7\(^{-/-}\) mice contained mixed C57BL/6J–129SvEv strains (refer to 2.1) rederived into 129SvEv mice. Since 4 genes on different chromosomes were altered in these functional
knock-out (KO) mice, litter mates could not be considered as appropriate controls. Rather, in this case, both age- and sex-matched C57BL/6J or 129SvEv WT mice were used.

A set of typical experiments using thymocytes from such mice are shown in Figure 4.7. Here, the time course of the DxS induced rises in thymocytes from the TRPC1,3,6,7−/− mice (aqua and light green for Ca²⁺ and Na⁺, respectively) typically lay between the time courses from C57BL/6J (Figure 4.7A & B, blue or green) and 129SvEv WT “controls” (mauve or olive). Specifically, in mice with a SvEv background, the rises were diminished and appeared delayed (Figure 4.7C; t_half for both Ca²⁺ and Na⁺; n = 6, p <0.01). However, the amplitudes of the DxS [Ca²⁺]_i rise was not significantly different between the TRPC1,3,6,7−/− and WT 129SvEv thymocytes (Figure 4.7D; n = 14, p ~0.3). Likewise, between the 129SvEv WT mice and the TRPC1,3,6,7−/− mice, the [Na⁺]_i rise was not significantly different either (Figure 4.7E; n = 14, p ~0.6). These experiments not only rule out TRPC6 as a candidate, consistent with the observations above, but also TRPC1, -C3 and -C7. The difference in both the Ca²⁺ and Na⁺ rises in mice with a 129SvEv background could indicate that the signalling leading to channel gating requires extended time for amplification and may suggest strain differences in the signalling strength.

Whilst TRPC1,3,6,7−/− mice allowed the exclusion of the respective TRPC channels, it does not rule out TRPC2 channels which are also activated by DAG. Therefore, I turned my attention to this channel.
Figure 4.7 DxS rises remain in TRPC1,3,6,7−/− thymocytes

(A) Time courses of DxS [Ca\(^{2+}\)] rises from a WT C57BL/6J (blue), a SvEv129TRPC1,3,6,7−/− (aqua) and a 129SvEv (mauve) mouse. DxS is added at \(t = 0\) min. (B) Same as in (A) but for [Na\(^{+}\)]. KO (light green), WT SvEv (olive) (C) Box-and-whisker plot showing the \(t_{\text{half}}\) in C57BL/6J (blue and green, controls) and 129SvEv strain mice (grey boxes, Ca\(^{2+}\) purple median bar, Na\(^{+}\) olive median bar) (D) Box-and-whisker plot of peak amplitudes of the [Ca\(^{2+}\)] rises was not significantly different between the 129SvEv (mauve) and 129SvEvTRPC1,3,6,7−/− (aqua, \(n = 14\)). (E) Same as in (D) but for [Na\(^{+}\)], 129SvEv (olive) and 129SvEvTRPC1,3,6,7−/− (light green, \(n = 14\)).

4.3.4.3 TRPC2 is unlikely involved either

Trpc2 has been shown to be highly expressed in mouse thymocytes (Figure 1.13; Inada et al. 2006). However, there is no evidence either for or against TRPC2 protein expression in these cells. To address this, I used ICC to check for it, using an Ab against an extracellular TRPC2 epitope (Table 2.4). Results from ICC flow cytometry experiments (\(n = 4\)) consistently showed low levels of anti-TRPC2 Ab
binding (Figure 4.8). The small shift in the population density toward to right upper quadrant in Figure 4.8(iv) perhaps indicates very low expression of TRPC2 protein on the PM. This data neither supports nor refutes a likely role for TRPC2.

![Figure 4.8 TRPC2 is poorly detected](image)

Typical contour plots showing expression of TRPC2 channel protein on the surface of CD8\(^{hi}\) thymocytes. This figure panel corresponds with those in Fig. 4.2. The legend for Fig 4.2 applies here as well.

Unfortunately, there is a current lack of specific TRPC2 antagonists. However, like for TRPC3, -C6 and -C7, the TRPC2 channel can also be blocked by 2-APB (50 µM; Lucas et al. 2003, Viitanen et al. 2013). Given that 2-APB actually caused a potentiation of the Dxs rises (see above), this data is inconsistent with TRPC2 playing a significant role in the Dxs rises.

**4.3.5 TRPA1 does not underlie the Dxs rises**

Since TRPA1 is predicted to be present in the PM (see Figure 4.2), I checked if the OAG-induced rise could be blocked by the selective TRPA1 antagonist HC030031 (2-(1,3-dimethyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-7-yl)-N-[4-(propan-2-yl)phenyl]acetamide). This is illustrated in Figure 4.9A & B. Compared with the OAG controls (Ca\(^{2+}\); black; Na\(^{+}\); grey), the addition of 20 µM HC030031 (IC\(_{50}\) ~6 µM; McNamara et al. 2007) with OAG at \(t = 0\) min (arrow) reduced both cation rises (aqua; \(n = 2\)). This finding confirms that TRPA1 channels are present in the
PM, but their activation does not account for all the OAG-induced cation influx. This suggests that other OAG-sensitive channels contribute to the rises.

Having evidence that TRPA1 channels can be activated in thymocytes, I investigated if it was involved in the DxS rises using 20 µM HC030031 or the more potent TRPA1 blocker A967079 (IC₅₀ rat ~0.29 µM; Chen & Hackos 2015). I note that the efficacy of these chemicals varies depending upon species, cell type and channel activation mechanisms (reviewed by Chen & Hackos 2015) and that their inhibitory effect has not been reported specifically for thymocytes.

Investigating the role of TRPA1, either 20 µM HC030031 or 1 µM A967079 ((NE)-N-[(E)-1-(4-fluoro phenyl)-2 methyl pent-1-en-3-ylidene] hydroxylamine) were added at 1 or 5 min prior to DxS, respectively. As shown in Figure 4.9C–E, preincubation with A967079 did not alter the DxS induced [Ca²⁺]ᵢ and [Na⁺]ᵢ rises (black trace and boxes; Ca²⁺: n = 4, pᵣ = 0.9; Na⁺: n = 4, pᵣ = 0.7). Similarly, in the presence of HC030031 (Figure 4.9E, orange boxes), the peak amplitudes of the [Ca²⁺]; (n = 4, pᵣ = 0.7) and [Na⁺]; rises (n = 4, pᵣ = 0.2) were not significantly changed. These findings suggest that TRPA1 is unlikely to be involved in the DxS rises.

To strengthen this argument even more, I checked if the DxS rise was absent in thymocytes from a TRPA1⁻/⁻ mouse. In such thymocytes, the Trpa1 mutation (Kwan et al. 2006) is presumed to cause the same loss of function as confirmed in neocortical pyramidal cells (Kheradpezhouh et al. 2017). Shown in Figure 4.9F, the DxS induced cation rise was still present in TRPA1⁻/⁻ thymocytes. This result strengthens the argument that TRPA1 channels are not involved. It leaves TRPV1 as the remaining DAG sensitive channel to be explored. While the OAG with added 2-APB results do not support the presence of this channel, it may be that the 50 µM OAG concentration was insufficient to activate TRPV1.
Figure 4.9 OAG activates more than one channel

(A) Time course plots of the [Ca\textsuperscript{2+}]\textsubscript{i} response when at \( t = 0 \) min either OAG (black); or OAG plus HC030031(aqua) were added. (B) Same as in (A) but for [Na\textsuperscript{+}]\textsubscript{i}. (C) Time course of DxS [Ca\textsuperscript{2+}]\textsubscript{i} in control (blue) and when A967079 is added (black arrow) 5 min prior to DxS (black trace). (E) As in (D) but for [Na\textsuperscript{+}] control (green). (E) Box-and-whisker plots summarising the effect of HC030031 (orange) and A967079 (black) on DxS Ca\textsuperscript{2+} and Na\textsuperscript{+} peak amplitude relative to the DxS control peak amplitude indicated by grey dashed line. (F) Time courses of Ca\textsuperscript{2+} and Na\textsuperscript{+} rises in TRPA1\textsuperscript{−} thymocytes.
TRPV1 antagonists do not block the rises

Hence, I investigated if selective blockers of TRPV1 abolished the DxS cation rises. Both, capsazepine (CPZ; N-[2-(4-chlorophenyl)ethyl]-7,8-dihydroxy-1,3, 4,5-tetrahydro-2-benzazepine-2-carbothioamide) and AMG9810 (3-(4-tert butyl phenyl)-N-(2,3-dihydro-1,4-benzodioxin-7-yl)prop-2-enamide) are blockers of TRPV1 (Amantini et al. 2017, Gavva et al. 2005). AMG9810 reportedly inhibits rat TRPV1 with an IC$_{50}$ of ~85 nM and here, I used it at 500 nM. The potency of CPZ as a TRPV1 blocker varies between species. In HEK-293 cells transfected with mTRPV1, Correll et al. (2004) reported that the IC$_{50}$ was ~1.4 µM. In thymocytes, Amantini et al. (2017) showed incubation with 1 µM CPZ was sufficient to inhibit TRPV1 function. Here, I initially chose 1.2 µM.

An experiment in which 1.2 µM CPZ was used is illustrated in Figure 4.10A (inset). Compared to the control for Ca$^{2+}$ (blue), the peak amplitude with 1.2 µM CPZ added 1 min before DxS shows an apparent inhibition (red). Notably the red trace starts from an elevated baseline, indicating that this concentration of CPZ likely caused a rise in [Ca$^{2+}$]. The control for CPZ alone added at $t = 0$ min (inset: black arrow/trace) confirms this suspicion. As a consequence, I reduced the CPZ concentration to 0.5 µM and checked if this prevented the rise in background [Ca$^{2+}$]. The results are presented in Figure 4.10A & B. Exposure to this concentration alone (black) did not elevate the background [Ca$^{2+}$] or [Na$^+$]. Additionally, when then exposed to DxS after pre-incubation for 1 min (red arrow), neither the DxS [Ca$^{2+}$] nor [Na$^+$] rises were inhibited (red, $n = 5$, $p_1 = 0.45$ and 0.65, respectively). While this result could indicate that TRPV1 channels are not involved, the apparent lack of block may simply be explained by having chosen an insufficient CPZ concentration.

To rule this possibility out, the more potent antagonist AMG9810 was used. Its addition to the suspension did not notably elevate background [Ca$^{2+}$]. (Figure 4.10C).
4.10C, black) and, when added 1 min prior to Dxs, it did not significantly block either the $[\text{Ca}^{2+}]$ (Figure 4.10C, grey, $n = 7$, $p = 0.6$) or $[\text{Na}^+]$ rise (Figure 4.10D, grey, $n = 7$, $p = 0.9$). These findings indicate that AMG9810 was also unable to block both rises and suggests that TRPV1 does not underlie the Dxs induced cation rises. This now means that all the DAG activated candidates TRPC6, TRPA1 and TRPV1 have been ruled out. Summarised in Figure 4.11, this finding, however, may point to the involvement of a channel activated by a metabolite of DAG such as arachidonic acid (AA).

![Figure 4.10 Dxs rises not due to TRPV1](image)

(A) Time courses of $[\text{Ca}^{2+}]$: when 0.5 µM CPZ was added at $t = 0$ min (black), or at $t = -1$ min (red) with Dxs added at 0 min (red trace) and control (blue). Inset: Same traces but plotted as $R$ indo-1 $\Delta F/F_0$ when 1.2 µM CPZ was added. (B) As in A, but monitoring $[\text{Na}^+]$. (C) Time courses of $R$ indo-1 $\Delta F/F_0$ in control (blue) with Dxs added at $t = 0$ min (grey arrowhead) and when incubated with AMG (grey), and AMG on its own (black). (D) Same as in (C) for $[\text{Na}^+]$ but without the AMG on its own.
Summary of the pharmacological screening steps used to exclude DAG activated channels from the DxS induced cation rise and direct the research toward the examination of channels that are activated by AA and/or are directly gated by mechanical stress.

Figure 4.11 Channels directly activated by DAG are excluded
4.3.7  

**Arachidonic acid and 5-LOX inhibition potentiate the rises**

Turning my attention to the DAG metabolite, AA, I checked if the cation rise was sensitive to this PUFA. In thymocytes the response to exogenously applied AA is varied. Khodorova and Astashkin (1994) showed that, the addition of 6 μM AA caused a rapid increase in \([\text{Ca}^{2+}]_i\) by ~100 nM, and Astashkin et al. (1993) found that 3 μM AA led to rapid intracellular acidification by ~0.2 pH units. Considering this, I used AA at 10 μM.

Such a set of experiments is illustrated in Figure 4.12A & B (black). The addition of 10 μM AA 1 min prior to DxS significantly potentiated the increase in \([\text{Ca}^{2+}]_i\); but not \([\text{Na}^+]_i\) (\(n = 3, p_t < 0.01\) and \(p_t = 0.76\), respectively). However, when I tested if there was a difference between the two extents of potentiation, I found there was none (\(n = 3, p_t = 0.54\)). This outcome is likely due to the small sample size and large variability seen within the data for Na⁺. Using the principle of parsimony, these two answers are best reconciled if there was an increase in both.

Interestingly, following the addition of 10 μM AA and DxS at \(t = 0\) min (Figure 4.12A, black), there was a rapid increase in \([\text{Ca}^{2+}]_i\), albeit small. While this early rise might reflect a direct activation of an AA- or pH-sensitive channel extraneous to the DxS \([\text{Ca}^{2+}]_i\) rise, the fact that both rises maintain a similar time course suggests AA potentiated the channel causing both rises.

In support of this idea, I found that the rises were also potentiated by the addition of 4 μM nordihydroguaiaretic acid (NDGA; 4-[4-(3,4-dihydroxy phenyl)-2,3-dimethylbutyl]benzene-1,2-diol). This chemical is reported to potently inhibit TRPM7 (IC₅₀ = 6.5 μM in HEK293 cells; Chen et al. 2010). However, it is also an inhibitor of 5-lipoxygenase (5-LOX; Papadogiannakis & Barbieri 1997) which, in T-lymphocytes, plays an important role in the metabolism of AA and formation of immunomodulatory leukotrienes (Cook-Moreau et al. 2007). Inhibition of 5-LOX is predicted to drive up endogenous AA levels and thus could provide a
mechanism for potentiating activation of an AA-sensitive channel involved in the DxS rises.

As shown in Figure 4.12D & E (grey), the addition of 4 µM NDGA at \( t = 0 \) min on its own did not elevate background \([Ca^{2+}]\). Rather, it slowly rose over 10 min, while \([Na^+]\) remained unchanged. It is therefore unlikely that this small elevation would have had an inhibitory effect on the cation rises. After pre-treatment with NDGA and compared to control, the \( Ca^{2+} \) rise was significantly potentiated, but not the one for \( Na^+ \) (Figure 4.12F, orange box-and-whisker plots; \( Ca^{2+} \): \( n = 5, p_t < 0.01; Na^+ \): \( n = 5, p_t = 0.69 \)). However, as found with AA, there was no difference between the two extents of potentiation \( (n = 4, p_t = 0.33) \). I note that in contrast to the case with AA, there was no immediate rise in \( Ca^{2+} \) suggestive of the idea that it takes time for AA to build up when the 5-LOX pathway is blocked, in contrast to when exogenous AA is added to the suspension. Both sets of data strongly suggest that the channel activated by the signalling cascade is modulated by the lipid contents in the membrane.
Figure 4.12 Arachidonic acid and NDGA both potentiate both rises

(A) Time courses of $[\text{Ca}^{2+}]_i$ under control conditions (blue) and when AA is added (black arrow) with DxS at $t = 0$ (grey arrowhead). (B) Same as in (A) but for $[\text{Na}^+]_i$. (C) Box-and-whisker plots summarising the potentiation by AA of both rises (black) relative to the respective control (dashed line). (D) Time courses for DxS $[\text{Ca}^{2+}]_i$ under control conditions (blue) and when 4 µM NDGA is added (orange) 1 min prior to DxS at $t = 0$ (grey arrowhead) and when 4 µM NDGA only is added at $t = 0$ (grey). (E) As in (D) but for $[\text{Na}^+]_i$. (F) Same as in (C) but for NDGA.
Table 4.3 Pharmacological properties of remaining candidate channels

Reported properties with references and where relevant concentrations.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Activated</th>
<th>Inhibited</th>
<th>Insensitive</th>
<th>Unknown</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>TRPV3 (Hu et al. 2006), TRPM2 (Hara et al. 2002) TRPV4 (indirectly; Watanabe et al. 2003b)</td>
<td>TRPM7 (Chen et al. 2010); Enhances Piezo1 inactivation (Romero et al. 2019)</td>
<td>TRPM3 (Rampino &amp; Hawy 2011)</td>
<td>TRPM6, TRPM6/7, TRPV2</td>
</tr>
<tr>
<td>2-APB</td>
<td>TRPV2, TRPV3, TRPM6, TRPM6/7 TRPM1 (? potentiate; Shen et al. 2009)</td>
<td>TRPM7 (50 µM) TRPM3 (100 µM; Xu et al. 2005)TRPM2 (30 µM; Togashi et al. 2008)</td>
<td>TRPV4</td>
<td>Piezo1</td>
</tr>
<tr>
<td>GsMTx4</td>
<td>Piezo1, TRPV2 (Zanou et al. 2015)a</td>
<td>TRPV4 (Miyamoto et al. 2014)</td>
<td>TRPM7</td>
<td></td>
</tr>
</tbody>
</table>

Given these observations, I refocussed my investigation towards remaining channels that are modulated by AA (or its metabolites) and 2-APB and blocked by GsMTx4. These channels are presented in Table 4.3. It is evident that none of these candidates has all the “right” properties. Only TRPV3 is known to be sensitive to both AA and 2-APB. While there is no evidence to suggest TRPV3 is directly mechanically gated, it could be indirectly mechanosensitive. For example, via loss of coupling to the cytoskeleton and/or a change in membrane curvature caused by alteration of the composition of lipids. Alternatively, it may be that the sequential activation of two channels is required. In this amended list of candidate channels, TRPV2, -M7, -V4 and Piezo1 are reportedly mechanosensitive, but of these, only TRPV2 is known to be both activated by 2-APB and inhibited by GsMTx4 (Zanou et al. 2015). Therefore, I examined if TRPV2 could be involved in the rises.
4.3.8 **TRPV2 is unlikely involved either**

*Trpv2* is highly expressed in thymocytes (Fig. 1.13; Inada *et al.* 2006). In addition, my ICC results in Figure 4.2 support the idea that the protein is expressed on the PM. To explore the involvement of TRPV2, I used the antagonist tranilast (2-[3-(3,4-dimethoxy phenyl)prop-2-enoyl-amino] benzoic acid) at 75 µM (Aoyagi *et al.* 2010, Sugio *et al.* 2017) and such an experiment is illustrated in Figure 4.13A & B. Specifically, I found the addition of 75 µM tranilast 1 min before that of Dxs had no effect on both the Ca\(^{2+}\) (*n* = 4, *p* > 0.9) and the Na\(^{+}\) rises (*n* = 4, *p* > 0.9). This data does not support involvement of TRPV2 channels.

Since TRPV2 is unlikely, the other mechanosensitive channels that need evaluating are TRPV4 and TRPM7. While the effect of GsMTx4 on TRPM7 is unknown, TRPV4 is reportedly insensitive to this blocker, which makes it an unlikely candidate.

![Graphs](image)

**Figure 4.13 TRPV2 in unlikely involved**

(A) Time courses of [Ca\(^{2+}\)]. with Dxs added at *t* = 0 min in control (blue) and when pre-incubated with tranilast for 1 min prior to adding Dxs (black). (B) Same as in (A) but for [Na\(^{+}\)].
4.3.9 TRPV4 is not involved either

Nevertheless I checked for its involvement using the antagonist GSK2193874 (3-((1,4’-Bipiperidin)-1’-ylmethyl)-7-bromo-N-(1-phenylcyclopropyl)-2-[3-(trifluoromethyl)phenyl]-4-quinolinecarboxamide, IC\textsubscript{50} = 5 nM; Thorneloe et al. 2012). I found that when 40 nM GSK2193874 was added 1 min after DxS (data not shown), neither the Ca\textsuperscript{2+} (n = 3, p\textsubscript{t} = 0.34) nor the Na\textsuperscript{+} rise (n = 4, p\textsubscript{t} = 0.83) were affected. This finding is consistent with the idea mentioned in the lead-in and suggests that TRPV4 does not appear to be involved.

The next channel to consider was therefore TRPM7.

4.3.10 TRPM7 is also an unlikely candidate

Trpm7 is very highly expressed in thymocytes, but distinct from TRPV2, it can be inhibited by 2-APB. In Jurkat cells, 50 µM 2-APB has been shown to partially inhibit TRPM7 (Prakriya & Lewis 2002), although the mechanism of inhibition may be indirect (Chokshi et al. 2012a, Pang et al. 2012). While the potentiation by 2-APB presented above (Figure 4.6C & D) is inconsistent with the properties reported for TRPM7, it cannot be ruled out that a heteromeric TRPM6/7 channel could be potentiated. Therefore, I evaluated if TRPM7 or TRPM6/7 when blocked with 10 µM NS8593 (IC\textsubscript{50} ~1.6 µM; Chubanov et al. 2012, Luongo et al. 2018) was involved in the rises.

I found that the addition of NS8593 at t = 1 min did not significantly reduce the amplitude of the [Ca\textsuperscript{2+}] and [Na\textsuperscript{+}] rises (Figure 4.14A & B, red; n = 5, p\textsubscript{t} = 0.42 and n = 5, p\textsubscript{t} = 0.34, respectively). To rule out the possibility that a block could only be achieved when the channel is open, NS8593 was added at t = 14 min. Still, the DxS rise was not reduced (n = 2). Notably, when added at t = 1 min, 10 µM NS8593 caused an early small Ca\textsuperscript{2+} increase, that interestingly did not inhibit the rises. However, preincubation with 15 µM NS8593 did abolish the rises (Figure 4.14C
& D, grey), but notably with this higher concentration there was an increased elevation of background $[\text{Ca}^{2+}]$ (Figure 4.14C, black). Therefore, this “block” of the DxS rise is unlikely attributable to TRPM7 channel block but rather to elevated background $[\text{Ca}^{2+}]$, similar to the block shown in 3.3.3 and 3.4.5.

This set of experiments also rules TRPM7 out and confirms the observation that a block of the rises can be caused by prior elevation of background $\text{Ca}^{2+}$. It also rules out TRPM6, which is potently inhibited by 10 µM NS8593 (Ferioli et al. 2017).

![Figure 4.14: Unlikely involvement of TRPM7 or TRPM6/7](image)

(A) Time courses of $[\text{Ca}^{2+}]$ with DxS added at $t = 0$ min in control (blue), with 10 µM NS8593 added 1 min (red) or 14 min after DxS (black). (B) Same as in (A) but for $[\text{Na}^+]$. (C) Time courses of $[\text{Ca}^{2+}]$ given as $R \text{ indo-1 } \Delta F/F_0$ with DxS added at $t = 0$ min for control (blue), when added at $t = 0$ min on its own (black) and after pre-incubation 5 min with 15 µM NS8593 (grey). (D) Same as in (C) but for $[\text{Na}^+]$ and after normalising for the baseline.

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The next step was to evaluate if TRPV3, TRPM1 or Piezo1 channels are involved. Common to all TRPV, some TRPM and Piezo1 channels is that they are sensitive to the hexacationic dye ruthenium red (RuR). I, therefore, considered if RuR could block the DxS rises.

4.3.11 **Ruthenium red blocks both rises**

RuR has a reported IC$_{50}$ of 0.12 µM for TRPV3 in mouse (Grubisha et al. 2014). While the IC$_{50}$ value for TRPM1 has not been determined, it can be blocked with 10 µM (Shen et al. 2009). Recalling from the Introduction (see Table 1.3), RuR has many molecular targets, including the mechanosensitive Piezo1 channel (IC$_{50}$ = 5.4 µM; Coste et al. 2012). Nevertheless, it is frequently used to investigate TRPV channel activity (Ahluwalia et al. 2002, Grubisha et al. 2014, Güler et al. 2002, Li et al. 2006, Peier et al. 2002, Watanabe et al. 2003a) and has, on one occasion, been used to block TRPM1 (Shen et al. 2009). I, therefore, checked if the DxS rises were sensitive to RuR and used it at 2.5 or 10 µM.

RuR causes an open channel block (Cibulsky & Sather 1999). Therefore, I added it after reaching the plateau phase of the cationic rise, when a maximum number of channels is open. Such an experiment is shown in Figure 4.15A & B. Compared to the control DxS rises, when 10 µM RuR ($n = 9$) was added at $t = 12$ min (red), both [Ca$^{2+}$]$_i$ and [Na$^+$]$_i$ started to decay, the former exponentially, the latter delayed. In both cases the block was incomplete. Since DxS is a polyanionic chemical binding to sites on the PM, it may be that in the presence of DxS the hexavalent cation RuR may not easily get access to the outside of the channel pore. To address this, I added it much earlier, specifically at $t = 1$ min after DxS. As shown in Figure 4.15A & B (grey), when added early, the rises were completely blocked. As seen several times before, this could have been the result of RuR elevating background [Ca$^{2+}$]. I therefore provide evidence that this was
not the case (Figure 4.15A, inset, pink). After adding 10 µM RuR on its own, the background [Ca$^{2+}$]$_i$ did not rise ruling this possibility out.

I wondered if a much lower concentration of RuR could also block the cationic rise. For this I chose 2.5 µM RuR. When it was added at $t = 12$ min (Figure 4.15C & D, black), only [Ca$^{2+}$]$_i$ was significantly affected and the decay became linear. Figure 4.15E provides a box plot which compares the reduction of both [Ca$^{2+}$]$_i$ and [Na$^+$]$_i$ associated with 10 and 2.5 µM RuR added at $t = 1$ and $t = 12$ min. In such experiments, the reductions in [Ca$^{2+}$]$_i$ were highly significant, regardless of time and concentration. The [Na$^+$]$_i$ reduction at 10 µM was also significant ($n = 7$, $p_t <0.0001$), but not for 2.5 µM added at $t = 12$ min (black/green box, $n = 9$).

This set of experiments shows that the DxS rise is caused by (an) element(s) that is/are sensitive to RuR as both 2.5 and 10 µM were able to block it. Notably, the 2.5 µM concentration is below the IC$_{50}$ reported for Piezo1 channels, but not for TRPV3.

Having already presented evidence in the Introduction, for high expression of 
Piezo1 (Figure 1.9) and Trpm1 (Figure 1.13 and Figure 1.15), I decided to check the expression of Trpv3 and Trpm1 in the ImmGen database for the αβT cell group.
Figure 4.15 Ruthenium red blocks both rises

(A) Time courses of the DxS $[\text{Ca}^{2+}]_i$ rise in control (blue) and after 10 µM RuR added at $t = 12$ min (red) or at $t = 1$ min (grey). Inset: Time course of $[\text{Ca}^{2+}]_i$: when 10 µM RuR was added on its own at $t = 0$ min (pink). (B) Same as in (A) but monitoring $[\text{Na}^+]_i$. (C) Time courses of $[\text{Ca}^{2+}]_i$ in control (blue) and after 2.5 µM RuR added at $t = 12$ min (black) or at $t = 1$ min (purple). (D) Same as in (C) but for $[\text{Na}^+]_i$. (E) Box-and-whisker plots summarising blocks for 2.5 (purple $n = 5$; black $n = 9$) and 10 µM (grey $n = 5$; red, $n = 9$) for $\text{Ca}^+$ and $\text{Na}^+$ relative to the DxS peak amplitude (dashed line, grey).

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4.3.12 TRPV3 and TRPM1 expression in thymocytes

The relative expression level of Trpv3 was examined by searching the αβT cell group in the ImmGen database. Shown in Figure 4.16A & B, there is an ~2-fold increase in the expression of Trpv3 and Trpm1 within the DP thymocyte subset. These data concur with the findings reported by Inada et al. (2006).

As there is no extracellular anti-TRPM1 Ab available, I only investigated the expression of TRPV3 protein in the PM using ICC. As shown in Figure 4.16C, a distinct shift in fluorescence intensity occurred when detecting the anti-TRPV3 Ab by staining with a FITC conjugated Ab (iv). This result is suggestive of the presence of TRPV3 protein. Additionally, the Ab was found to be sensitive to immunogen, as indicated by the low detection when preincubated with the blocking peptide (iii).

I next explored if TRPV3 was involved in the cation rise. TRPV3 is an unusual thermosensitive channel in that it does not open readily. Rather its activation can be sensitised with repeated thermal stimuli or by co-stimulation by dissimilar agonists acting at different sites (Liu et al. 2011, Zhang et al. 2019). Such sensitisation has been shown to produce a biphasic time course (Chung et al. 2005). Specifically, channel activation is possible at subthreshold temperatures (<33°C) if synergistically exposed to 2-APB (Chung et al. 2004). Distinct from TRPV3, TRPM1 is not known to be temperature-sensitive.
Figure 4.16 Expression of TRPV3 and TRPM1

(A) mRNA expression of *Trpv3* as provided for the ImmGen database abT cell group; Probe set:10378367. In descending order, the four DP populations enclosed by the red dashed lines are: all; blasts; small resting; and CD69+ early positive selection. The subpopulations framed by the dashed black lines are DP 69- preselection, DP 69+ early positive selection and DP early positive selection. (B) Same as in (A) but for *Trpm1*. Probe set:10553861. (C) Typical contour plots from ICC experiments used to detect TRPV3 protein in the PM of CD8^hi^ thymocytes.

4.3.13 2-APB at 30°C restores the Dxs [Ca^{2+}]_i rise

To check for the functional involvement of TRPV3, I investigated if the Dxs cation rise could be activated at subthreshold temperature (30°C) in the presence of 70 µM 2-APB. The failure of DP thymocytes to respond to the addition of Dxs when the cell suspension was maintained at 30 rather than 37°C is shown in Figure
4.17A & B (aqua). However, the addition of 70 μM 2-APB prior to the DxS at this temperature indeed brought the rises back (pink, as compared to blue/green recorded at 37°C). Consistent with sensitisation at 30°C, there was a longer delay to $t_{\text{half}}$. Overlaying the two time courses, I found that under these conditions, the Na⁺ rise slightly preceded that of Ca²⁺ suggestive of a channel letting predominantly Na⁺ in (Figure 4.17C and inset). This did not show up in the pooled data as there was no difference between $t_{\text{half}}$ and rate of Ca²⁺ and Na⁺ ($n = 3$, $p_t = 0.85$ and 0.93, respectively) suggestive of the fact that the variability between samples hid this subtle difference. Even though it looks as if the rise in A was potentiated compared to the control at 37°C, but that in B was not, this observation is made with caution, as the relevant comparator would be a rise at 30°C, which unfortunately could not be obtained. Most likely, this reflects that the regulation of the Ca²⁺ homeostasis at the two temperatures is likely very different. In accordance with the finding reported by Xu et al. (2002), the mechanism of 2-APB sensitisation at 30°C could also be blocked by the addition of 10 μM RuR at $t = 1$ min (Figure 4.17A & B, black). The amount of potentiation against three different 2-APB concentrations at 37°C is given in Figure 4.17D. Additionally, the case for sensitisation at 30°C is given (pink), which is the justification for using 70 μM in these experiments. These data strongly suggest that TRPV3 activation could very likely contribute to DxS induced cation rises. It may suggest that in this case, this channel predominantly lets Na⁺ through and a significant amount of the concomitant Ca²⁺ rise is likely due to NCX in reverse mode.
Figure 4.17 2-APB sensitisation at 30°C rescues both cation rises

(A) Time courses of \([Ca^{2+}]_i\) under control conditions at 37°C (blue), at 30°C (aqua), when 2-APB was added at 30°C at \(t = -1\) min (pink) and in the presence of RuR (black) added at \(t = 1\) min. (B) Same as in (A) but for \([Na^+]_i\). (C) Overlay of both cation rises with the same colour code as in (A) and (B). Inset: blow-up to reveal that the early Na\(^+\) rise precedes that of Ca\(^{2+}\). (D) Dose-response relationship between \([Ca^{2+}]_i\) rise and 2-APB concentration at 37°C (black). The values are joined by a polynomial spline with the dashed line indicating the normalised \([Ca^{2+}]_i\) rise. The pink dot at 70 µM outside the joined dots was obtained for the case of sensitisation at 30°C.
4.3.14 Extracellular ATP potentiates the cation rises

Having previously found that 100 µM ATP on its own did not reproduce the Ca\textsuperscript{2+} rise (Feakes 2012), I quickly checked if the DxS rises might be sensitive to ATP as described by Doerner et al. (2011) for TRPV3. Shown in Figure 4.18A & B, the addition of 100 µM ATP potentiated both the Ca\textsuperscript{2+} and Na\textsuperscript{+} rise. This finding further supports the argument that TRPV3 likely underlies the DxS rises.

Figure 4.18 Extracellular ATP potentiates the DxS rise

(A) Time course plot of [Ca\textsuperscript{2+}]: under control conditions (blue) and when 100 µM ATP was added with DxS at $t = 0$ min (black). (B) As for (A) but for [Na\textsuperscript{+}].

Having previously concluded that the channel involved while not directly activated by DAG, was likely gated by its metabolite, Figure 4.19 provides a flow chart of the pharmacological investigations that lead to TRPV3 being decided as the most likely candidate underlying the DxS induced cation rise.
**Figure 4.19 Pharmacology indicates TRPV3 likely underlies the Dxs rises**

Summary of the flow of pharmacological experiments which lead to the conclusion that TRPV3 activation underlies the Dxs rises. Notably, mechanosensitive TRP channels are shown not to play a role in the Dxs cation rise. Rather activation of the mechanosensitive Piezo1 channels is predicted to occur at the initiation of the signal transduction.
4.4 Discussion

4.4.1 Summary

The results presented in this chapter indicate that several TRP channels show a high level of mRNA expression and, using ICC, are likely co-expressed in the PM of thymocytes. In addition, I show that some of them can be activated and blocked by agonists and antagonists, a fact which likely confounded the pharmaceutical exploration presented. In addition to what was presented in Chapter 3, I found that the channel(s) underlying the DxS rises is/are 1) unlikely activated by DAG, but rather by a downstream signalling molecule, most likely a lipid like AA or its metabolite as the latter potentiated the rises. 2) In addition, I found that the rises were also potentiated by 70 µM 2-APB, a property that excludes a number of TRP channels listed in Table 3.2. 3) The cation rises are blocked by ≥2.5 µM RuR, 4) 100 µM ATP potentiated the rises and 5) 2-APB at 30°C rescues the rises, a temperature at which normally no rise can be elicited. These features are known characteristics of the channel TRPV3 and suggest that this channel likely underlies the DxS rises.

4.4.2 mRNA expression as a guide to channel expression

With scarce evidence of TRP channels in thymocytes, the level of gene expression in the various thymocyte populations was reviewed. In this thesis, I have referred to the BioGPS and ImmGen databases. The former provides information on the expression level in broad DP and SP thymocyte populations relative to a wide range of other tissue and cell types, while the latter more specifically compares expression levels between multiple distinct subpopulations of immature and mature αβT cells. Understanding that the correlation between mRNA and protein expression is loose (Schwanhausser et al. 2011, Tian et al. 2004), it is nevertheless assumed that where a marked increase in mRNA transcription
occurs, the level of protein expression is also high. However, several times, this assumption led the research toward wrong candidate channels.

4.4.3 ICC detection in the PM

The ICC results indicated the anti-TRPC6, -C7, -A1, -V2, and -V3 antibodies were, in general, sensitive and specific to their respective immunogen which corresponded to an epitope in an extracellular domain of the protein. Moderate off-target binding was apparent with the anti-TRPV4 Ab (Figure 4.2iii, bottom row). Also, despite its high mRNA expression, TRPC2 was poorly detected by ICC (Figure 4.8). These results suggest that either the antibody was insufficiently sensitive to the immunogen, the epitope was hidden, or as found in primary erythroid cells (Chu et al. 2004), detection of TRPC2 in the PM is negligible. In mice, expression of the TRPC2 protein appears predominantly in the vomeronasal organ and testis. Its trafficking to the PM may be impaired dependent upon splice variation (Hofmann et al. 2000).

Considering the negligible level of Trpc7 mRNA reported by Inada et al. (2006, Fig. 1.13), the detection of the anti-TRPC7 Ab was not anticipated (Figure 4.2iv, row 2). This could be explained by delayed protein degradation, or alternatively could indicate “off-target” Ab binding to surface protein(s) that contain a similar epitope. If indeed TRPC7 is present, this highlights the issue of using mRNA data to extrapolate to channel protein presence in these cells. While not conclusive, the incomplete block by 10 µM Gd³⁺ and 80 µM 2-APB of the OAG rises could suggest the presence of the Gd³⁺-insensitive TRPC7 channel, which is inhibited at considerably higher concentration (100 µM; Okada et al. 1999).

Acknowledging that variation between batches occurs, I note that the anti-TRPC6 and -TRPA1 antibodies have been validated by others (anti-TRPC6; Diez-Bello et al. 2019, anti-TRPA1; Sullivan et al. 2015). In this study the TRPC1,3,6,7⁺ and
TRPA1<sup>−/−</sup> mice used were functional KOs with a mutation within the channel pore. Not unexpectedly, I found the proteins were still detected, albeit with a lesser intensity (data not shown).

4.4.4 Activation of TRP channels in the PM

Besides ICC, I was able to use “specific” activators of some TRP channels as a means to detect if they were functionally expressed in the PM and if they were involved in the DxS rises.

4.4.4.1 TRPA1

I confirmed the presence of DAG sensitive channels, specifically TRPA1 and likely TRPC7 using the activator OAG, a DAG analogue, together with specific channel blockers. The partial block of the OAG-induced cation rises by HC030031 and A967076 (as well as by 10 μM Gd³⁺) is consistent with TRPA1 channels in the PM. However, TRPA1 does not underlie the DxS rise. I showed that the DxS rise was still present in thymocytes from a TRPA1<sup>−/−</sup> mouse.

Also present in the OAG rise is a Gd³⁺ insensitive channel which, based on the 2-APB block, is more likely TRPC7 than TRPV1 (see below).

4.4.4.2 TRPC6

TRPC6 can be activated by 5 μM hyperforin (Leuner et al. 2007). This chemical also caused a non-selective cation rise, consistent with the presence of TRPC6 channels in the PM. However, I found no evidence that supported involvement of TRPC6 in the DxS rises. Neither adding the TRPC6 antagonists norgestimate (Miehe et al. 2012) nor SKF96365 (He et al. 2017, Inoue et al. 2001), at 9 μM and 10 μM, respectively, blocked the DxS rises. Furthermore, instead of blocking TRPC6 channels, 70 μM 2-APB (Lievremont et al. 2005) potentiated the rises.
Strengthening this argument, and also providing evidence against involvement of TRPC7, -C3, and -C1, I showed that the DxS rises persisted in thymocytes from TRPC1,3,6,7−/− mice.

4.4.5 Exclusion of other candidate channels

I have also tested for other candidate channels proposed in Table 3.2. However, for most of these, it was not possible to find suitable agonists. Consequently, I used mostly “specific antagonists” to see if they affected the DxS rises. It is important to note that during the time of this research, I learnt that a lot of specific antagonists were rather “unspecific”. As a consequence, in some instances and where possible, I used more than one blocker and based my deductions on the internal consistency between the two. Lacking expression systems for the various TRP channels, unfortunately, in many instances, it was not possible to provide positive controls. I do not think that this is a major issue as overall, the final conclusion is not based on the result of a single antagonist but rather on the consideration of a combined set of features.

4.4.5.1 TRPC2

Unfortunately, there are no selective blockers for this channel since in humans, TRPC2 is a pseudogene. This channel was ruled out on the basis that it should have been inhibited by 2-APB (Zhang et al. 2010).

4.4.5.2 TRPM2

As with TRPC2, the 2-APB potentiation of the DxS rises does not support involvement of TRPM2. This channel is potently inhibited by 30 µM 2-APB (Togashi et al. 2008), however using this concentration I found the DxS rises were not notably changed (Figure 4.17D). Additionally, both PIP2 (Tóth & Csanády 2012) and intracellular Ca2+ (Csanády & Törőcsik 2009) are obligate co-activators
of TRPM2. Therefore, my results that showed the DxS induced cation rises were significantly reduced when PLC activation was blocked (Figure 3.10A & B) and when background [Ca$^{2+}$] was elevated do not favour TRPM2 involvement.

4.4.5.3 TRPM3

Having no specific TRPM3 channel blocker, it was ruled out as a likely candidate based on my 2-APB and edelfosine results (Figure 4.6A & B and Figure 3.10C & D). Specifically, Xu et al. (2005) have shown 100 µM 2-APB potently blocks TRPM3, which is contrary to the potentiation I observed with 70 µM 2-APB. Furthermore, like TRPM2, TRPM3 activation is dependent upon PIP$_2$ as a cofactor (Badheka et al. 2015) and hydrolysis of PIP$_2$ by PLC activity acts to negatively regulate TRPM3 (Tóth et al. 2015). Therefore, my finding that PLC inhibition significantly reduced the DxS rise, also suggests TRPM3 involvement is not likely.

4.4.5.4 TRPM6/7

These channels were excluded as the inhibition with 10 µM NS8593 (Chubanov et al. 2012, Ferioli et al. 2017) did not block the cation rises either. In addition, although most likely present in thymocytes (Jin et al. 2008a), the involvement of TRPM7 was not supported by the 2-APB potentiation nor by addition of the 5-LOX inhibitor NDGA, a reported potent inhibitor of TRPM7 (Chen et al. 2010).

4.4.5.5 TRPV1

The evidence for TRPV1 expression in the PM of DP thymocytes is not strong (Amantini et al. 2017, Amantini et al. 2004, Farfariello et al. 2012). But, since this channel is commonly co-expressed with TRPA1 (Schwartz et al. 2011, Staruschenko et al. 2010, Zhou et al. 2013), it seemed prudent to clarify that TRPV1
was indeed not involved in DxS rises. I found the addition of AMG9810, a potent selective TRPV1 blocker, did not reduce the rises.

4.4.5.6 TRPV2

Similarly, exposure to 75 µM tranilast (Aoyagi et al. 2010) did not notably reduce the DxS rises. Additionally, while not definitive, inhibition of the DxS [Ca$^{2+}$]$_i$ and [Na$^+$]$_i$ rises by edelfosine (a PLC inhibitor, Figure 3.10A & B) and by LY294002 (a PI3K inhibitor, Figure 3.15A & B) does not support involvement of TRPV2. As depletion of PIP$_2$ reportedly has a crucial role in TRPV2 desensitisation (Mercado et al. 2010), I would expect the DxS rises to remain unchanged or perhaps potentiated when PIP$_2$ hydrolysis was impeded. This was not the case.

4.4.5.7 TRPV4

The involvement of TRPV4 was tested using 40 nM GSK2193874 (Thorneloe et al. 2012) and again, the DxS rises persisted. I note that the Ca$^{2+}$ influx detected in ConA stimulated T cells was shown by Majhi et al. (2015) to require TRPV4 and TRPV1 activation. In agreement with Tellam and Parish (1987) I found in DP thymocytes stimulated with DxS, the addition of ConA at $t = 20$ min (Figure 3.2Figure 3.1A) caused an additive [Ca$^{2+}$]$_i$ rise that was indicative of activation of another Ca$^{2+}$ permeable channel. Whilst I did not further investigate the channel(s) associated with this additive [Ca$^{2+}$]$_i$ rise, it perhaps indirectly provides evidence against TRPV4 and TRPV1 involvement in the DxS [Ca$^{2+}$]$_i$ rise.

Based on Table 3.2, Piezo1 was the remaining channel that is directly activated by mechanical force.

4.4.6 RuR abolishes the rises

Interestingly, concentrations >2.5 µM abolished the DxS rises when added at $t = 1$ min. However, when added at $t = 12$ min after DxS, the block was incomplete.
This observation may be explained by steric hindrance between the polyanionic DxS and the polycationic RuR. Nevertheless, the RuR block is highly suggestive of an involvement of a member of the TRPV family (Ahluwalia et al. 2002, Grubisha et al. 2014, Güler et al. 2002, Peier et al. 2002, Watanabe et al. 2003a), but does not rule out that other channels may also be blocked by it. Notably, TRPM1 (Shen et al. 2009) and Piezo1 are also sensitive to RuR (Coste et al. 2012).

4.4.7 2-APB at 30°C rescues the DxS rises

Having provided evidence in Chapter 3 that the DxS rises were abolished at temperatures ≤30°C, I have shown here that 70 μM 2-APB can rescue the rise at this subthreshold temperature. This sensitisation by 2-APB is a known and unique feature of TRPV3 (Chung et al. 2005, Chung et al. 2004) strongly suggesting that this channel underlies both cation rises.

In 3.6.7 (p. 207), I proposed that a thermally activated STIM1/Orai1 sustained Ca\(^{2+}\) influx might act to negatively regulate TRPV3 activation. Supporting this proposal, it is notable that, in STIM1/Orai1 transfected HEK293 cells, 50 μM 2-APB inhibits the thermally activated Ca\(^{2+}\) influx (Liu et al. 2019). Perhaps concurrent inhibition of STIM1/Orai channels and subthreshold activation of TRPV3 channels by 2-APB explains why the DxS cation rises not only recovered at subthreshold T but were also potentiated.

4.4.8 TRPV3 as most likely candidate

By process of elimination, I have arrived at TRPV3 as the most likely candidate based largely on two characteristics, namely its subthreshold sensitisation at 30°C by 2-APB plus its block by RuR, which in the presence of Ca\(^{2+}\) remains incomplete. The fact that elevated background [Ca\(^{2+}\)]\(_i\) can inhibit cation permeation also supports this argument as raised Ca\(^{2+}\) increases Ca/CaM-binding to TRPV3 leading to channel inhibition (Phelps et al. 2010). In addition, this
channel also activates upon PIP2 depletion and is potentiated with 100 µM ATP (Doerner et al. 2011)

Despite these considerations, I cannot fully exclude the possibility that TRPM1 or Piezo1 may be involved (see below). I note that both TRPV3 and TRPM1 are both “neglected” entities in their respective families (Irie & Furukawa 2014, Nilius et al. 2014). Additionally, Piezo1 has been known for about 15 years, but only recently has received much attention.

4.4.8.1 Conductance and ion selectivity

Among all TRPV channels, TRPV3 apparently has by far the largest single channel conductance (Yang & Zhu 2014). The question is if its partial conductance is sufficiently large to bring about the observed Na\(^+\) rise. This may indeed be the case as the channel may undergo pore dilation to allow even NMDG\(^+\) to permeate (Chung et al. 2005, Xiao et al. 2008), with some uncertainty remaining about how pore-dilation affects ion selectivity (Chung et al. 2005). Furthermore, there are reports in the literature that at 25 and 39°C, the conductances are 201 and 337 pS (Chung et al. 2004), respectively.

In addition to the Ca\(^{2+}\) entry, the large Na\(^+\) conductance of this channel is well suited to maintain a high [Na\(^+\)]\(_i\) for a considerable time as the extrusion/sequestration of Na\(^+\) using pumps is very much smaller (Hille 2001). This [Na\(^+\)]\(_i\) then drives a rise in [Ca\(^{2+}\)]\(_i\) for an equally long time, by bringing in additional Ca\(^{2+}\) via transport activation. As well, activation of counter currents facilitates restoration of the resting membrane potential and, in doing so, increases the electrical driving force for Ca\(^{2+}\) entry through TRPV3.

Originally, TRPV3 was described as a channel with considerable Ca\(^{2+}\) permeability (Peier et al. 2002, Smith et al. 2002, Xu et al. 2002). However, more recent data indicates that this may not be the case. In fact, bovine TRPV3 show a
large single channel conductance for Na+ at physiological potentials (≈73 pS; Schrapers et al. 2018). There are several known splice variants in mice (Smith et al. 2002, Wang 2006, Yang & Zhu 2014), but unfortunately, most of them have not been investigated in regard to ion-selectivity or conductance remains to be investigated in the most variants. However, it has been found that colocalization of a short variant, which cannot form a functional channel, with functional TRPV3 channels significantly increases the channel activity (Wang 2006).

Whether or not TRPV3 has an additional permeation pathway, that activates following channel hysteresis, remains to be investigated. However, I note that at least for TRPM3, two ion permeation pathways have been described. The canonical pore had a partial conductance for both Na+ and Ca2+ with strong rectification in the physiological range (Vriens et al. 2014). The non-canonical pathway had a much larger conductance and loss of rectification.

4.4.8.2 Ca2+ and Mg2+ sensitivity

High-affinity channel inhibition has been described for Ca2+-binding to an area around Asp-641 in the pore loop area (Luo et al. 2012). In addition, this site is also important for RuR binding. Once Ca2+ is bound, RuR is in competition for channel block. This is consistent with my observation that once the Ca2+ had reached a plateau, the block by RuR was incomplete.

However, Ca2+ together with CaM can also affect the channel from the intracellular side by forming a Ca2+-CaM complex. Since this complex maintains the channel in a low activity state (Xiao et al. 2008), this could be the explanation for the observation that elevated background Ca2+ blocked the channel. This also occurs in a realistic concentration as the first Ca2+ binding to CaM is in the range of ~100 nM; i.e. just around the resting background [Ca2+]. (Persechini & Cronk 1999). Notably, the Ca2+-CAM inhibition of TRPV3 is attenuated in response to
repeated stimulation leading to channel sensitisation (Phelps et al. 2010, Xiao et al. 2008).

It is known that Mg\(^{2+}\) can inhibit TRPV3 also both from the intra- and extracellular sides. It apparently does this by reducing the unitary conductance with little influence on the open probability, again with Asp-641 (see above) identified as the site of action on the extracellular side. With 10 mM Mg\(^{2+}\), a channel block of ~60\% was seen (Luo et al. 2012), largely consistent what I saw in my data. Unfortunately, no data for higher concentrations was given. The intracellular binding site is in the C-terminus of the channel (Luo et al. 2012).

### 4.4.8.3 Thermosensitivity

TRPV3 is a channel sensitive within the warm range (~31 – 39°C, Peier et al. 2002), but there are reports using fast temperature changes that on its own, temperature activates this channel >54°C (Yao et al. 2011). However, its sensitivity is increased at depolarised voltages (Xu et al. 2002), with increased Ca\(^{2+}\) influx at a high rate of temperature change, if repeatedly stimulated (Liu & Qin 2017, Xiao et al. 2008) and if exposed to plant alkaloids like camphor and citral (Stotz et al. 2008) or the chemical 2-APB (Chung et al. 2004). This suggests that in contrast to other thermo-sensitive channels, its activation may reflect some “coincidence detection”, whereby within the warm range, at least two factors may have to cooperate to lead to channel opening. This “sensitisation”, consistent with what I have found, seems to be a unique feature of TRPV3.

### 4.4.8.4 Mechanosensitivity

Results in Chapter 3 indicated that the DxS induced [Ca\(^{2+}\)]\_i and rises [Na\(^{+}\)]\_i were sensitive to GsTMx4. To the best of my knowledge, mechanosensation is not an explicit property of TRPV3. However, I would like to point out that in a recent review Liu and Montell (2015) suggested that “most TRPs are actually
mechanosensitive channels, which undergo conformational changes in response to tension imposed on the lipid bilayer, resulting in channel gating.” Consequently, mechanosensation may not be a distinctive feature as first assumed.

4.4.8.5 Presence in the membrane

Upon Ca\(^{2+}\) influx, a number of channels can be inserted into the membrane quite quickly (reviewed by Montell 2004). For example, during neurite growth, in response to epidermal growth factor stimulation, TRPC5 channels are rapidly inserted in the PM \((t_{1/2} = 49 \pm 2.4 \text{ s}, \text{ Bezzerides et al. 2004})\). Furthermore, these authors showed the PI\(_3\)K dependent insertion was inhibited by LY294002. The delay in the onset of the DxS rises is consistent with the idea that TRPV3 channels may not be in the membrane and require insertion first. However, based on the data showing that elevated background [Ca\(^{2+}\)]\(_i\) blocks the rises, such a scenario is unlikely. In addition, insertion from the endosome would preclude mechanosensation. Consequently, TRPV3 channels are very likely already present in the PM. This idea, however, does not rule out that its rate of insertion or removal may not be affected by the signalling cascade and/or the Ca\(^{2+}\) rise.

4.4.8.6 PIP\(_2\) depletion

Even though PIP\(_2\) is a universal channel modulator (reviewed by Hilgemann et al. 2001, Ramsey et al. 2006), in the case of TRPV3, its hydrolysis potentiates the channel (Doerner et al. 2011) via an interaction in the C-terminus. In this light, the experiment with DAG might have increased PIP\(_2\) availability, but at the same time, its activation of PKC may have occluded the expected inhibition of the channel.
4.4.8.7 Potentiation by extracellular ATP

Another feature consistent with TRPV3 is that the influx was potentiated using 100 µM ATP. This has also been seen Doerner et al. (2011). This feature further adds to the growing body of evidence that TRPV3 is the most likely channel.

4.4.8.8 Lipid modulation

It has been reported that polyunsaturated acids like AA are capable of potentiating TRPV3 (Hu et al. 2006) consistent with what I saw. In addition, 5-LOX inhibition also showed a potentiation. This is most likely due to the fact that when 5-LOX is blocked, the concentration of AA in the PM increases.

4.4.8.9 Homo- vs. heteromeric channels

It has been reported that TRPV3 and TRPV1 channels may form heteromeric channels (Smith et al. 2002). In HEK293 cells, these channels exhibit unique activation properties that derive from both proteins and are influenced by subunit stoichiometry (Cheng et al. 2012). While these proteins are co-expressed in neural cells (Brown et al. 2013), there is no evidence confirming that such a heteromeric configuration exists in thymocytes or related lymphocytes.

4.4.9 Alternative 1: TRPM1

The attractiveness of TRPM1 lies with the fact that it is associated with a multimolecular signalling complex containing GRP179, which has a binding site for HS (Orlandi et al. 2013, Orlandi et al. 2018). TRPM1 sensitivity to 2-APB has so far not been reported, however it shares the block by RuR (Shen et al. 2009). Notably, this channel has a large partial conductance for Na⁺, which also makes it very attractive in this instance (Oancea et al. 2009). While activation by PIP₂ depletion remains to be clarified, PIP₂ hydrolysis and DAG generation indirectly modulate the channel. Specifically, DAG-dependent activation of PKC removes
an inhibitory Mg\(^{2+}\) block of TRPM1 (Rampino & Nawy 2011). While TRPM8 and more recently TRPM2, -M3, -M4 and -M5 are reportedly thermostensitive (Held et al. 2015, Tan & McNaughton 2016, Tominaga 2007), to my knowledge this feature has not been reported for TRPM1. Consequently, despite several attractive features, TRPM1 involvement in the cation rises is not very likely.

4.4.10 Alternative 2: Piezo1

This channel shares a number of properties with TRPV3, among them mechano-sensation and block by RuR (Coste et al. 2012). The block by 2.5 µM RuR is half of the IC\(_{50}\) for this channel suggesting that it is unlikely that this channel is involved as the conduit to cation permeation. Piezo1 channels are potently inhibited by GsMTx4, however it is important to note that the inhibition is not direct. Rather the gating of the channel is modified by GsMTx4 insertion into the lipid bilayer altering lipid composition and tension transduction to the channel (Suchyna et al. 2004). The reason to keep this channel in consideration is because it quickly activates and inactivates (Gottlieb & Sachs 2012, Wu et al. 2017b, Zhao et al. 2016). Because of this, there is a possibility that this channel could have activated immediately upon Dxs addition and escaped detection. Because it can pass a significant amount of Ca\(^{2+}\), it then could have kick-started the signalling cascade by priming LFA-1. Without data during the gap, it is futile to speculate about Piezo1 involvement any further.

4.4.11 Conclusion

After excluding a considerable number of channel candidates from several families and based on the pharmacological properties uncovered, I am concluding that the most likely candidate gating the cation rises observed in DP thymocytes upon stimulation with Dxs is TRPV3. However, I have not been able to test this idea in KO mice. In contrast to the outset of this project thinking that
DxS caused a Ca\textsuperscript{2+} rise, I have also come to the conclusion that there is a much larger concurrent Na\textsuperscript{+} rise present in these cells. Without further quantification of the size of the Na\textsuperscript{+} rise and involving realistic simulations, I may be tempted to speculate that a significant component of the Ca\textsuperscript{2+} rise may in fact be driven by the accumulation of Na\textsuperscript{+}.

In the next chapter, possible physiological and immunological implications will be explored
5 Physiological and immunological implications

Ca\(^{2+}\) is without doubt a pivotal messenger in a myriad of cellular functions. In T cell development it plays a decisive role in regulating the migration, proliferation, differentiation, apoptotic death by instruction or neglect, and selection of thymocytes. Discreet Ca\(^{2+}\) flickers, increased oscillations and large global rises have been shown to effect thymocyte migration, arrest and sustained stromal cell–thymocyte interaction, respectively (Bhakta & Lewis 2005, Bhakta et al. 2005, Bunnell et al. 2011, Melichar et al. 2013, Ross et al. 2014, Wei et al. 2009). Furthermore, it appears that the amplitude of the [Ca\(^{2+}\)] \(i\) rise associated with positive selection events may reflect the “strength” of TCR–pMHC engagement, and with recurring TCR–pMHC interactions there is an associated elevation in [Ca\(^{2+}\)]. (Fu et al. 2013, Ross et al. 2014). I propose that in some DP thymocytes undergoing early positive selection, the [Ca\(^{2+}\)] \(i\) rise induced by TCR signalling may be elevated by Ca\(^{2+}\) entering synergistically through TRPV3 channels. Dependent upon repeated stimulation, this non-selective cation channel is activated by a signalling cascade triggered by the CD8\(\beta\) coreceptors interacting with HS on stromal cells. Notably, this mechanism is restricted to thymocytes in which the CD8 coreceptor is minimally sialylated.

For this thesis, I examined a Dxs induced signalling pathway that leads to a sustained [Ca\(^{2+}\)] \(i\) rise of ~160 nM. This pathway is specific to a subpopulation of DP thymocytes. Even though this [Ca\(^{2+}\)] \(i\) rise has been known for over 30 years, the molecular elements that underpin this rise remained unknown. As it is hypothesised that the Dxs [Ca\(^{2+}\)] \(i\) rise may mimic a similar rise recorded in preselection DP thymocytes during rosette formation with cTEC\(^{hi}\), I am interested in understanding the mechanisms that lead to this Ca\(^{2+}\) influx. Furthermore, I am particularly intrigued by the signalling pathway activated following CD8\(\beta\)
binding DxS, this interaction inducing the \([\text{Ca}^{2+}]\) flux that normally occurs in preselection \(\alpha\beta\text{TCR DP thymocytes during their adhesion to cTEC}^{hi}\) (Simon Davis 2015).

Notably, the \([\text{Ca}^{2+}]\) rise is dependent upon either DxS or HS binding to CD8\(\beta\) on preselection DP thymocytes (Simon Davis 2015). The addition of DxS is proposed to cross-link and stimulate multiple surface receptors, including at least both CD8\(\beta\) and LFA-1. However, adding an equivalent amount of heparin to a thymocyte suspension does not reproduce the rise, this discrepancy probably being due to heparin having a much lower mol. wt. than DxS \((i.e., 10–15\text{ kDa vs. } 500\text{ kDa})\) and thus unable to cross-link CD8 molecules as effectively as DxS (Simon Davis 2015). Also, unlike DxS, heparin has not been shown to bind LFA-1 (Vermot-Desroches et al. 1991) however, during cell–cell interactions, LFA-1 would likely be activated by its endogenous ligand ICAM-1.

Altogether, my results from Chapters 3 and 4 indicate that the channel underlying the DxS induced \([\text{Ca}^{2+}]\) rise is 1) a non-selective cation channel with a large partial conductance for \(\text{Na}^+\), 2) thermosensitive and sensitised by 2-APB at subthreshold temperatures, 3) mechanosensitive, 4) blocked by increased background \([\text{Ca}^{2+}]\), 5) activated downstream of PLC-\(\gamma\)1 or -\(\beta\)2, 6) not gated by either IP\(_3\) or DAG, 7) potentiated by AA, 2-APB and ATP, 8) enhanced by PKC and/or PKA phosphorylation, and 9) blocked by RuR.

Perhaps excluding direct mechanical gating, TRPV3 is a channel that has all these properties. The functional and immunological implications of this conclusion will be discussed below.
5.1 Functional implications

The process of selection is rigorous and markedly costly, with only 3–5% of DP thymocytes maturing to the SP phase of development (Merkenschlager et al. 1997). Within the large pool of preselection DP thymocytes (88.39 ± 60·10⁶) ~66% will die by neglect because they express nascent αβTCRs that do not sufficiently engage with pMHC molecules (Sawicka et al. 2014). Of the remaining DP thymocytes, ~91% will express αβTCRs that strongly bind pMHC complexes. To prevent maturation of likely autoreactive T cells, excessively avid αβTCR–pMHC interaction activates negative selection signalling that triggers SOCE mechanisms to cause apoptosis and clonal deletion.

In contrast, preselection DP thymocytes expressing αβTCR that bind pMHC molecules, with the bond duration and strength reaching an optimal range, receive positive selection signals. These activated DP thymocytes continue to undergo further interactions with stromal cells as they make their way back to the medulla. Increasingly stringent testing of the TCR–pMHC bond strength ensures tolerance to self-peptides as they mature to become SP thymocytes. The outcome of rigorous selection is the generation of a pool of peripheral T cells; with an average of 2 x 10⁶ (Casrouge et al. 2000) and 2 x 10⁷ (Naylor et al. 2005) clones in mice and humans, respectively.

5.2 A proposed role for TRPV3

A synergistic Ca²⁺ influx via TRPV3 activation may provide a mechanism that 1) enhances positive selection of thymocyte clones that risk death by neglect due to unsatisfactory MHC engagements and 2) fine tune selection of clones that border on self-reactivity. Specifically, activated downstream of MHC-I–CD8–HS binding, this Ca²⁺ rise could synergistically augment TCR/CD3 dependent Ca²⁺ signalling and influence selection outcome, thus refining the repertoire of T cells.
Figure 5.1 & 5.2 illustrate the proposed role of TRPV3 when TCR–pMHC-I binding is absent, suboptimal or super-optimal.

5.3 Without TCR–pMHC-I interaction

In preselection thymocytes, MHC-I–CD8 binding without TCR engagement has been suggested to promote death by neglect (Grebe et al. 2004). Where the TCR fails to engage with the MHC (Figure 5.1A), I suggest CD8 signalling activated by binding to both MHC-I and HS (augmented during rosette formation on cTEC^{hi}) may hasten the deletion of such T cell clones. In fact, I propose that without TCR engagement, TRPV3 channel activation and the resultant sustained rise in Na\(^+\) (green) and Ca\(^{2+}\) (blue) triggers “death by instruction” signalling.

5.4 With sub-optimal TCR–pMHC-I interaction

In contrast, when thymocyte clones express TCRs that sub-optimally engage pMHCs, activation of TRPV3 may play an important role in prolonging their survival by adding to a basal [Ca\(^{2+}\)]\(_i\) rise of ~250–550 nM (Figure 5.1C). Depicted in Figure 5.1D, initial CD8\(^{\beta}\)–MHC–HS binding (black dashes) stabilises suboptimal TCR–pMHC binding (red dashes) and initiates signalling that enables ongoing survival of clones that otherwise will die by neglect. Repeated transient TCR–pMHC evoked Ca\(^{2+}\) entry (red) summates with the TRPV3 dependent Ca\(^{2+}\) influx (blue). While the concomitant Na\(^+\) influx likely helps to maintain the Ca\(^{2+}\) rise. Positively selecting cells have been shown to increase their background [Ca\(^{2+}\)]\(_i\) overtime (Nakayama et al. 1992, Ross et al. 2014). Therefore, despite suboptimal TCR/CD3 signalling, this synergistic Ca\(^{2+}\) entry may eventually lead positive selection of these clones. Notably, the identity of this other Ca\(^{2+}\) channel remains unclear.
Figure 5.1 TRPV3 enhances the $[\text{Ca}^{2+}]_i$ rise evoked by TCR/CD3 signalling
(A) Without TCR–pMHC engagement synergistic signalling downstream of HS–CD8β and ICAM1–LFA-1 interaction results in TRPV3 activation. (B) Dependent upon the strength and duration of HS–CD8β interaction (black dash), activation of TRPV3 channels leads to sustained concomitant [Na+] and [Ca²⁺] rises sufficient to promote ‘death by instruction’ signalling pathways. (C) In suboptimal TCR–pMHC–CD8 engagement, HS–CD8β binding synergistically enhances the signalling cascade. The [Ca²⁺] rise is within the range for promotion of positive selection. (D) Concurrent CD8β–HS induced TRPV3 Ca²⁺ entry (blue) adds to a [Ca²⁺] rise (red) evoked by suboptimal TCR–pMHC interaction and avidity (dark red dash). Increasing CD8β sialylation (orange triangle) progressively impedes the strength and duration of HS–CD8β interaction (black dash). TRPV3 concomitant supporting Na⁺ rise (green). (E) Concurrent with overly moderate TCR–pMHC–CD8 engagement, HS–CD8β binding synergistically enhances the signalling cascade. The [Ca²⁺] rise exceeds the range for positive selection. (F) The amplitude of Ca²⁺ transients evoked by TCR–pMHC interaction and avidity (dark red dash) is increased by the elevated basal [Ca²⁺] due to TRPV3 activation, to exceed the positive selection limit (black line).

Crucially, the TRPV3 activation mechanism is curtailed as the thymocyte matures. Progressively sialylation of CD8β in response to thymocyte activation, sterically hinders MHC-I–CD8–HS binding and beneficially decreases the sensitivity of the TCR–pMHC allowing the clone to undergo more stringent testing as it returns to the medulla. Activation of this mechanism in this population is predicted to expand the repertoire of the peripheral T cell pool at the lower (weakly binding) end as illustrated in Figure 5.2.

5.5 With moderate TCR–pMHC-I interaction

Finally, shown in Figure 5.1E & F is a proposed mechanism for redirecting selection in a population of preselection thymocytes when the TCR–pMHC-I bond strength is near the self-reactive/negative selection threshold. Notably, this population is thought to be small as activation of TRPV3 mechanism will only occur if CD8β sialylation is low and where background [Ca²⁺] is not already elevated in response to prior activation of the TCR/CD3 signalling complex. In these preselection thymocyte clones, concomitant activation of TRPV3 (downstream of MHC-I–CD8–HS stimulation) and the unknown Ca²⁺ permeable channel (activated by TCR/CD3 signalling) could rapidly raise background [Ca²⁺].
so that it exceeds the upper limit for positive selection signalling which is reportedly >600nM (F. black line). Whether the signalling pathways activated by such a \([\text{Ca}^{2+}]\) increase now direct differentiation pathways leading to “specialised” T cells development or instead promote a negative selection outcome remains to be investigated. Inhibition of the TRPV3 mechanism in this population of thymocytes may result in the unwanted export of T cells, that verge on being self-reactive, to the peripheral pool.

**Figure 5.2 Proposed selection tuning with TRPV3 activity**

Activation of TRPV3 in preselection thymocytes alters the range of thymocyte survival. In suboptimal TCR–pMHC binding, TRPV3 activity will promote positive selection (green striped). TRPV3 activity in cells which bind with moderate/strong force will fail positive selection (black striped).
5.6 Possible implications to disease

TRPV3−/− mice do not have an overt T cell phenotype (Bertin & Raz 2016). However, this observation does not rule out possible subtle changes to the range of the T cell repertoire. This remains to be investigated.

I have shown that the activation of TRPV3 was inhibited in conditions where background [Ca²⁺] was already raised by at least 60 nM. Interestingly, thermally activated STIM/Orai1 has been shown to raise background [Ca²⁺], and therefore could potentially inhibit TRPV3 activation during preselection thymocytes—cTEC hi interactions. As activation of the inhibitory Orai1 evoked Ca²⁺ influx occurs as temperatures fall from 39 to 37°C, perhaps fever spikes in this range during childhood results in adverse changes in the peripheral T lymphocyte repertoire.

As indicated in Figure 5.2, I proposed inhibition of TRPV3 during thymopoiesis results in a shift in positive selection, increasing the likelihood of maturation of thymocytes that verge on being self-reactive. Having these clones in the peripheral T cell pool, would potentially increase the chance of developing an autoimmune disorder if TRPV3 channels were to reactivate as a consequence of reduced surface sialylation.

Furthermore, inhibition of TPRV3 during thymopoiesis would narrow the range of survival of clones found at the lower end of TCR avidity scale (Figure 5.2). Preselection thymocytes with poor TCR–pMHC binding capability may not sufficiently increase [Ca²⁺], and so fail to be positively selected. Loss of these potentially beneficial T cells clones from the peripheral pool may result in a weaker immune system where activation of the immune system response may be poorer.
A reduced TCR repertoire may result in less effective recognition when rare harmful endogenous or exogenous antigens are presented. In humans, this might be associated with an increased susceptibility to infection, particularly with ageing (Naylor et al 2005), and perhaps a poorer prognosis in some cancers (Cui et al 2018). While it is interesting to note that inflammatory skin disorders are linked to TRPV3 dysfunction (Wang et al 2017), to my knowledge, association of these disorders with an altered T cell repertoire has not been studied.

Sialylation of CD8 in mature inactive mouse T cells impedes the mechanism I believe leads to TRPV3 activation. Notably, following mitogenic activation of Jurkat and mouse splenic T cells, Majhi et al. (2015) showed at least a 2-fold increase in TRPV1 >TRPV4 >TRPV3 >TRPV2 in the PM. However, while these authors detected activation of TRPV1 and TRPV4 they did not detect activation of TRPV3 downstream of either ConA or anti-CD3/anti-CD28 Ab activation. Perhaps because the stimuli provided did not lead to sensitisation of TRPV3, or as I have shown, a prior increase in [Ca^{2+}] would have an inhibitory effect on TRPV3. Nevertheless, following T cell activation, I propose CD8 de-sialylation could re-enable the mechanism that leads to TRPV3 activation.

Sensitisation of TRPV3 by repeated multiple stimuli, including AA, inflammatory mediators and fever, would enable a sustained Na^{+} and Ca^{2+} influx. Whether such an increase in background [Ca^{2+}] enhances Ca^{2+} cellular functions such as proliferation of activated T cells or cytotoxic activity, remains to be explored. Importantly, activation of such a mechanism would be curtailed by sialylation of CD8 with inactivation of the inflammatory response.

This raises the question as to what would happen if CD8 sialylation levels were pathologically impaired. Significantly, in mature T cells, TCR/CD3 expression level is higher (Guidos et al. 1990) and their aggregation is thought to enable amplification of TCR/CD3 signal strength. If combined with unimpeded HS–
CD8β–MHC-I binding and subsequent TRPV3 activation, I propose the resultant Na⁺ and Ca²⁺ influx could prime the TCR sensitivity, in doing so, increase the response in some clones to self-antigen–MHC complex ligation. In conditions of acquired idiopathic autoimmune disorders, perhaps triggered by viral infection, investigation of T cell sialylation state could perhaps prove interesting. As TRPV3 protein has been reported in human peripheral T cells (Majhi et al. 2015) perhaps this channel might present a novel therapeutic target in the future management of autoimmune disorders linked with abnormal sialylation.

5.7 Concluding remark

Throughout the course of this project, my thinking about selection shifted from involving Ca²⁺ rises to concomitant rises of both Na⁺ and Ca²⁺ and finally to a large Na⁺ influx together with a small Ca²⁺ rise. Given that Na⁺ rises have not received much attention in immunology and since in several systems Na⁺-dependent signalling has been described (Rishal et al. 2003, reviewed by Rose & Verkhratsky 2016, Verkhratsky et al. 2017), I would not be surprised if in the near future, such signalling outcomes may be uncovered.
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Reference list for Table 3.2


6 Appendices

6.1 Appendix 1: Heparan sulfate binding proteins

List of proposed HSBP that show an increase in expression in the αβ DP thymocyte populations. The functions of these proteins are described in the Immunological Genome Project (M. musculus) database (ImmGen) [http://www.immgen.org/databrowser/index.html](http://www.immgen.org/databrowser/index.html). In general, data available on the ImmGen website has been derived by profiling gene expression on Affymetrix microarrays using cells from five-week-old C57Bl/6J mice. The DP thymocyte populations of interest are described in the “abT cell” data group.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Title</th>
<th>Proposed function: from ImmGen data base and, as described in the protein knowledge database UniProtKB: <a href="https://www.uniprot.org/uniprot/">https://www.uniprot.org/uniprot/</a></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cd8b1</td>
<td>CD antigen, β chain 1</td>
<td>Coreceptor binds MHC class I; T cell signalling pathway</td>
</tr>
<tr>
<td>Cd4</td>
<td>CD4 antigen</td>
<td>Coreceptor binds MHC class II; T cell signalling pathway</td>
</tr>
<tr>
<td>Fas</td>
<td>TNF receptor family member</td>
<td>Receptor activity MAPK signalling pathway, mediator of apoptosis</td>
</tr>
<tr>
<td>Adgre5 (Cd97)</td>
<td>Adhesion G protein coupled receptor (GPCR) E5</td>
<td>Transmembrane signalling receptor</td>
</tr>
<tr>
<td>Insr</td>
<td>Insulin receptor</td>
<td>Protein kinase activity; Negative regulation of protein phosphorylation. In response to insulin leads to activation of PI3K-AKT/PKB signalling pathway</td>
</tr>
<tr>
<td>Anxa2</td>
<td>Annexin A2</td>
<td>Phospholipase inhibitor activity; Ca(^2+) dependent PIP(_2) binding; raft assembly. Ca(^2+) regulated membrane-binding protein who affinity for calcium is greatly enhanced by anionic phospholipids. May cross link PM phospholipids with actin and the cytoskeleton</td>
</tr>
<tr>
<td>Anxa5</td>
<td>Annexin A5</td>
<td>Ca(^2+) dependent phospholipid binding; receptor tyrosine kinase binding</td>
</tr>
<tr>
<td>Ptprc (Cd45)</td>
<td>Protein tyrosine phosphatase receptor type C</td>
<td>T cell receptor signalling pathway; interacts with Src family kinases. Acts as a positive regulator of T cell coactivation</td>
</tr>
<tr>
<td>Cd47</td>
<td>Rh-related antigen</td>
<td>Cell adhesion; co-stimulates T cell activation. Has a role in cell adhesion and modulation of integrins, may play a role in integrin dependent signal transduction</td>
</tr>
<tr>
<td>Lrrc8b</td>
<td>Leucine rich repeat regulating anion channel subunit B</td>
<td>Key player in regulation of cytosolic calcium, make act as leak channel in the ER (Ghosh et al. 2017)</td>
</tr>
</tbody>
</table>

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6.2 Appendix 2: Expression of HSBP in the abT cell group

mRNA expression levels of HSBP in the abT cell group as made available in the Immunological Genome Project (M. musculus) database (ImmGen). In general, data provided by the ImmGen website has been derived by profiling gene expression on Affymetrix microarrays using cells from five-week-old C57Bl/6j mice. The DP thymocyte populations of interest are described in the “abT cell” data group. [http://www.immgen.org/databrowser/index.html](http://www.immgen.org/databrowser/index.html). In descending order, the four DP populations framed by the dashed black lines are All; Blasts; Small resting; 69+ Early Positive selection. The three DP subpopulations framed by the dashed red lines are DP 69- preselection, DP 69+ early positive selection and DP early positive selection.
6.3 Appendix 3: Previously investigated channels

The following channels were investigated to determine if they were required in facilitating the Ca\(^{2+}\) influx observed following the addition of DxS to a thymocyte suspension. In the case of all target channels, in the presence of the inhibitory or activating chemicals and DxS, there was no significant change in the time course or amplitude of the DxS induced [Ca\(^{2+}\)] \text{rise} when compared to DxS only treated samples. None of these channels appeared to have a crucial role in facilitating the DxS induced [Ca\(^{2+}\)] \text{rise}.

<table>
<thead>
<tr>
<th>Target</th>
<th>Chemical</th>
<th>µM</th>
<th>antagonist</th>
<th>agonist</th>
</tr>
</thead>
<tbody>
<tr>
<td>VDCC</td>
<td>Cd(^{2+})</td>
<td>200</td>
<td>Ca(<em>{v})1.1, Ca(</em>{v})1.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ca(<em>{v})1.3, Ca(</em>{v})2.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ni(^{2+})</td>
<td>30</td>
<td>Ca(<em>{v})2.3, Ca(</em>{v})3.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ca(_{v})3.3</td>
<td></td>
</tr>
<tr>
<td>CNGA</td>
<td>dequalinium</td>
<td>1</td>
<td>CNGA1, CNGA2</td>
<td></td>
</tr>
<tr>
<td>P2XR</td>
<td>NF449</td>
<td>1</td>
<td>P2x1</td>
<td>P2x7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Suramin</td>
<td></td>
<td>10</td>
<td>P2x1</td>
<td>P2x7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPADs</td>
<td>10</td>
<td></td>
<td>P2X1,2,3,5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100</td>
<td></td>
<td>P2x7</td>
<td></td>
</tr>
<tr>
<td>ATP</td>
<td>100</td>
<td></td>
<td></td>
<td>P2xR</td>
</tr>
<tr>
<td>IP(_{3})R (PM)</td>
<td>TEA</td>
<td>20 mM</td>
<td>IP(_{3})R3</td>
<td></td>
</tr>
<tr>
<td>iGluR</td>
<td>D-AP5</td>
<td>50</td>
<td>NMDAR</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NBQX</td>
<td>20</td>
<td>AMPAR</td>
<td></td>
</tr>
<tr>
<td>nAChR</td>
<td>atropine</td>
<td>30</td>
<td>nAChR(\alpha)9, nAChR(\alpha)8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>nicotine</td>
<td>100</td>
<td>nAChR(\alpha)9, nAChR(\alpha)10</td>
<td>nAChR(\alpha)4</td>
</tr>
</tbody>
</table>
6.4 Appendix 4: Phylogenetic relationship between TRP genes

Schematic illustration of the phylogenetic relationship between TRPC, TRPV, TRPA1 and TRPM. Bar represents approximate 10% variation. Adapted from Minke (2006), and Nilius et al. (2014)
### Appendix 5: Commonly used chemicals

<table>
<thead>
<tr>
<th>Chemical (Cat. No.)</th>
<th>Sourced from</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine serum albumin (BSA)</td>
<td></td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>Merck Pty. Ltd. Vic. AUS</td>
</tr>
<tr>
<td>D-glucose</td>
<td>Gibco BRL Life technologies</td>
</tr>
<tr>
<td>Donkey serum</td>
<td>Santa Cruz Biotechnology, Inc Dallas TX 75220</td>
</tr>
<tr>
<td>Dimethyl sulfoxide (DMSO)</td>
<td>Sigma-Aldrich Gmbh St Louis, MO, USA</td>
</tr>
<tr>
<td>F15 Minimum essential media (MEM) powder # 41500-018</td>
<td>lot 1649212x Gibco® Life technologies Carlsbad, CA</td>
</tr>
<tr>
<td>Hydrochloric acid (HCl)</td>
<td>Diggers Australia Pty. Ltd. Maddington WA 6109</td>
</tr>
<tr>
<td>HEPES</td>
<td>Sigma Aldrich Gmbh St Louis, MO, USA. Santa Cruz Biotechnology, Inc Dallas TX 75220</td>
</tr>
<tr>
<td>KCl</td>
<td>Merck Pty. Ltd. Vic. AUS</td>
</tr>
<tr>
<td>MgCl₂·6H₂O</td>
<td>Ajax Chemicals Pty. Ltd. NSW AUS</td>
</tr>
<tr>
<td>NaCl</td>
<td>Merck Pty. Ltd. Vic. AUS</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>Merck Pty. Ltd. Vic. AUS</td>
</tr>
<tr>
<td>Na₂HPO₄·H₂O</td>
<td>Ajax Chemicals Pty. Ltd. NSW AUS</td>
</tr>
<tr>
<td>NaOH</td>
<td>Ajax Chemicals Pty. Ltd. NSW AUS</td>
</tr>
<tr>
<td>Paraformaldehyde (SLP1627)</td>
<td>ScienceLab.com Inc. Houston TX 77396 USA</td>
</tr>
</tbody>
</table>
6.6 Appendix 6: Orai/STIM gene expression profile

From the Immunological Genome Project (M. musculus) (ImmGen) database. The DP thymocyte populations of interest are described in the “abT cell” data group. The four DP populations enclosed by the red broken lines are in descending order: All; Blasts; Small resting; 69+ Positive selection transitional intermediate. In the DP populations Orai2 has the highest expression levels. Although notably, there is an ~3-fold increase in Orai1 expression in the “69+ Positive selection transitional intermediate DP” (green) and “DP 69+ early selection” (yellow) population. The three DP subpopulations framed by the dashed black lines are in descending order DP 69- preselection, DP 69+ early selection, DP early selection. The overall relative transcription level is indicated by the arbitrary numbers below each plot. [http://www.immgen.org/databrowser/index.html](http://www.immgen.org/databrowser/index.html) Probesets: 10525464; 10534570; 10557754; 10555681; 10521950